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COMPARISON OF ANALYTICAL METHODS USED FOR THE CHARACTERIZATION OF CARBONYL CONTENT OF FATS

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(Received: 6 October 1978; accepted: 19 March 1980)

The analytical methods commonly used for carbonyl determination are reviewed here. Based on the evaluation of the literature, three methods have been adapted for the comparison of quality of various rape seed oils. The total carbonyl content has been assayed spectrophotometrically while both spectrophotometry and gas chromatography have been used for the determination of the volatile carbonyl content of the samples.

The carbonyl index, which is proportional to the total carbonyl content of the sample, can be used to compare different raw and refined rape seed oils. The spectrophotometric volatile carbonyl assay can be used to detect the differences between raw and refined oils. Gas-chromatographic analysis of the volatile carbonyls allows for the detailed investigation of the individual carbonyls and the evaluation of the raw, refined and stored oils.

Foods, including cooking fats, are traditionally subjected to sensory analysis with regard to both flavor and taste. Partial or complete replacement of the sensory evaluation methods has been intensively investigated over the past decades. Both recent technical developments and the appearance of automated analytical instruments accelerated the replacement of the sensory evaluation methods.

The taste and flavor of oil originate from characteristic compounds inherently associated with the particular oil and from the decomposition products of both the original fat and its contaminations. According to the ways of their formation, the volatile compounds present in fats can be classified (PREVOT, 1971) as follows.

– Compounds originating from the natural flavor constituent of the oil. The concentration of these compounds in the refined oil is very low.

– The second group of the volatile compounds is formed in the refined oil itself by the so-called flavor reversion mechanism. It is assumed that even slight oxidation of the refined oil results in the formation of volatile compounds. These compounds are believed to be formed by decomposition from the oxidized products not removed in the refining step, and not, as thought before, by oxidation following the deodorization.

– The third group of the flavor compounds is formed by autoxidation.

Hydroperoxides, the primary products of autoxidation are mostly tasteless compounds devoid of any characteristic odor. However, their decom-

position products, the so-called secondary products are responsible for the rancid smell and taste of the oxidized fats. The most important oxidized products are, as far as flavor and taste go, the carbonyl compounds. A series of alkanals, alkenals and alkyldienals can be derived from the various hydroperoxides of the fatty acids. It can be assumed that the carbonyl content is correlated with the organoleptic character of the particular fat, and that the correlation is better than that observed for the peroxide number. Apparently, this assumption is straightforward, since the peroxide number indicates only the amount of tasteless and odorless peroxides, indirectly connected only with the changes of the taste.

At the analysis of the secondary products, the natural flavor components and the flavor reversion materials are generally also present.

Carbonyl analysis is customarily carried out along two main lines (PIERGIOVANNI & VOLONTERIO, 1976). The first approach calls for the physicochemical separation of the components followed by their identification by instrumental methods (gas chromatography, mass spectrometry, *etc.*). The second approach makes use of a suitable reagent to separate all the carbonyl compounds followed by the analysis of the individual derivatives.

The methods based on the first approach generally require oil samples of large quantities. DEBRUYNE (1964), *e.g.* subjected 90 l oil to steam distillation to remove the volatiles. The volatile fraction thus collected was repeatedly chromatographed and the fractions were identified by infrared spectrometry. Starting with 250 l soybean oil, CHANG and SMOUSE (1967) could identify as many as 71 components by infrared spectroscopy and mass spectrometry.

Since the handling of large samples posed special problems, the need for reduced sample sizes increased. SELKE and co-workers (1970) used only 100 ml oil. The volatiles were separated and analysed by mass spectrometry. In 1971, PREVOT and co-workers (1971) could decrease the sample size to as little as 100 μl by injecting it directly onto a gas chromatograph. A 5-cm long precolumn packed with uncoated support served as a guard column before the analytical column. Apart from the small sample size, the method had another advantage: there were no volatile losses since the original oil sample was directly injected onto the column. DUPUY and co-workers (1976) described a similar system. 500 mg of the oil sample tested was injected onto a precolumn packed with glass wool. They claimed that the total peak area of the chromatogram correlated sufficiently well with the taste scores. The method was proposed for the rapid instrumental characterization of the taste of vegetable oils.

BLUMENTHAL and co-workers (1976) investigated several deep-fry fats and oils. Volatiles were separated by freeze-drying in high vacuum and subsequently analysed by gas chromatography. The total peak area of the chromatogram and the taste scores correlated reasonably well.

The most common reagent used in the methods based on the second approach is 2.4-dinitro phenylhydrazine (DNPH). The reasons for the popularity of this reagent are the characteristic melting point of the DNPH derivatives, their long-term stability and good solubility in both medium and low polarity solvents.

POOL and KLOSE (1951) applied a chromatographic fractionation step prior to the determination of the individual DNPH derivatives. HENICK and co-workers (1954) also reported on a method for the determination of the DNPH derivatives in the presence of fats.

CHANG and KUMMEROW (1955) modified the carbonyl determination method. This improved technique allowed for the determination of taste reversion and the degree of rancidity of cooking oils. They reported a good correlation between the carbonyl number and the organoleptic scores of oil samples stored for various intervals.

FRANZKE and BAUMGARDT (1973) proposed the determination of the heptanal number as a rapid means of characterization of the total carbonyl content.

The recovery, separation and identification of the volatile components are extremely time-consuming and complicated tasks, because

– the concentration of the volatiles in the fat sample is always very low,

– along with the flavor compounds a number of other volatiles are also present in the samples,

– the flavor compounds form a mixture and their boiling point range is wide,

– the low concentration levels call for increased precision and sophistication,

– the solvents used may introduce artifacts while the frequently labile original volatiles can decompose in the course of the work-up process.

The primary aim of the work reported here has been the development of analytical methods yielding results compatible with those of the sensory evaluation. Detailed analysis of the volatile components is beyond the scope of this work. Instead, it concentrates on the comparison of various rape seed oils. Based on the above literature review, three methods have been selected and adapted.

1. Materials and methods

Carbonyl determination was carried out on conventional rape seed oils containing 50% erucic acid. The raw and refined rape seed oils were intermediate and final products of edible oil processing.

1.1. Spectrophotometric determination of the total carbonyl content

The method of HENICK and co-workers (1954) has been adapted. However, the amount of both the fat sample and the reagents has been decreased and the composition of the KOH solution modified. The method is based on the spectrophotometric determination of the 2,4-DNPH derivatives in an alkaline solution.

Chemicals:

– carbonyl-free benzene,

– carbonyl-free abs. ethanol,

– 0.05% (w/w)2,4-dinitro phenylhydrazine solution (the DNPH reagent was purified by repeated recrystallization from benzene at 50 °C),

– 4.3% (w/w)trichloro-acetic acid in benzene,

– 4% (w/w)KOH in ethanol (15 cm^3 28% (w/w)KOH in distilled water is brought to 100 cm^3 by adding ethanol. The dilute solution is freshly prepared daily).

Procedure:

0.02 g rape seed oil is weighed in a 10-cm^3 volumetric flask, 1 cm^3 4.3% (w/w) tricholoro-acetic acid solution, 1 cm^3 0.05% DNPH reagent solution, and 1 cm^3 benzene are added to it. An identical blind is prepared simultaneously. The solutions are thermostatted for 30 min at 60 °C, cooled to ambient temperature, allowed to stand for another 30 min, then 3 cm^3 4% KOH in alcohol is added. The flask is brought to mark with abs. ethanol. The absorbance at 460 nm is read on a suitable spectrophotometer after 10 min.

The results are calculated as described by CHANG and KUMMEROW (1955). The absorbance per 100 g oil is the so-called carbonyl index:

$$\text{Carbonyl index} = A_{460} \frac{100\ (g)}{\text{weight of sample}\ (g\,l^{-1})}$$

where A_{460} is the absorbance read at 460 nm.

1.2. Spectrophotometric determination of the volatile carbonyl content

The method of FIORITI and co-workers (1975) developed for fat-rich dried food products has been adapted. The method calls for the separation by steam distillation of the volatile carbonyls followed by their spectrophotometric determination as DNPH derivatives, analogous to the method for determination of total carbonyl content.

Chemicals:

– 0.1% (w/w)2,4-dinitro phenylhydrazine solution (prepared by dissolving the calculated amount of doubly recrystallized 2,4-DNPH in 0.1 *N* HCl at 40 °C, on a water bath).

– 4% (w/w) alcoholic KOH solution (prepared according to Section 1.1).

Procedure:

A mixture of 5 g rape seed oil and 30 cm^3 distilled water is agitated for 2 min in a high-speed mixer (3000 rpm). The emulsion is charged into a micro-*Kjeldahl* apparatus. The first 25 cm^3 of the distillate are condensed in an ice-cooled receiver. Ten cm^3 of the 0.1% (w/w) 2,4-DNPH reagent are added to an Erlenmeyer flask followed by 10 cm^3 of the distillate. The mixture is thermostatted at 60 °C for 30 min, cooled to ambient temperature and mixed with 20 cm^3 4% alcoholic KOH solution. After 10 min, the absorbance at 460 nm is read on a spectrophotometer.

The volatile carbonyl index is defined as the absorbance at 460 nm per 100 g oil. The average of 4 parallel determinations is used.

1.3. Gas-chromatographic characterization of the volatile carbonyls

The methods of BLUMENTHAL and his co-workers (1976) as well as of DUPUY (1976) have been adapted.

The arrangement of the pre- and the analytical column has been modified and the sample size decreased. 20μl of the oil tested is injected into the precolumn packed with glass wool. Then the precolumn is inserted into the evaporator. The analytical column is maintained at 50 °C for 10 min. During this period the volatile carbonyls are transferred to the initial section of the analytical column. After 10 min, the precolumn is removed from the evaporator and the regular chromatogram is obtained by temperature programing.

Gas-chromatographic conditions:

Pye Unicam 104 gas chromatograph.

Oven temperature: isothermal for 10 min at 50 °C, 10 °C min^{-1} program from 50 °C to 200 °C, isothermal for 30 min at 200 °C.

Evaporator: 150–170 °C.

Detector: 260 °C.

Analytical column: 4.0 mm I. D. × 2.4 m packed with 5% (w/w) OV-101 on Gas Chrom Q (80–100 mesh).

Precolumn: 3.0 mm I. D. × 100 mm packed with silanized glass wool.

Carrier gas: 40 cm^3 min^{-1} nitrogen,

FID gases: 30 cm^3 min^{-1} hydrogen,

400 cm^3 min^{-1} air.

2. Results and discussion

2.1. The total carbonyl content

The carbonyl index determined spectrophotometrically characterizes the total carbonyl content of rape seed oil, the major factor influencing the taste. The determination is carried out in the presence of the oil triglycerides,

without the separation or enrichment of the carbonyl compounds. The reproducibility of the method is shown in Table 1 summarizing the test results of a variety of rape seed oils. The sensory evaluation of the oils tested was carried out according to HUNGARIAN STANDARD (1977). The expected value (M) of sensory quality ($\bar{x}$) at a probability level of 95% and n = 8 is $M = \bar{x} \pm 0.83$.

Taste evaluation according to the Standard prescribes the following scores:

Uncharacteristic pleasant taste	10
Pleasant, light rape seed taste	9
Characteristic, light rape seed taste	8
Characteristic, but not strong rape seed taste	7
Characteristic rape seed taste free of exogenous taste	6
Exogenous, unpleasant rancid taste	0–5

The largest standard deviation for four parallel determinations was 5.3%. The analytical conditions applied were not suitable for the characterization of raw rape seed oil since $A_{460} > 0.8$.

Table 1

The carbonyl index of refined rape seed oil samples (four parallel determinations)

Sensory scores (average ± standard deviation)	Carbonyl index (average ± standard deviation)	Coefficient of variation (%)
6.0 ± 0.15	15.05 ± 0.43	±2.9
7.8 ± 0.22	12.78 ± 0.18	±1.4
8.0 ± 0.25	9.65 ± 0.45	±4.7
6.5 ± 0.20	13.35 ± 0.62	±4.6

The relationship between the taste scores obtained by sensory evaluation and the spectrophotometric carbonyl index of fresh and stored rape seed oil samples is plotted in Fig. 1.

The average taste score value of the commercial rape seed oils produced in 1977 and 1978 was 6.9, the minimum being 6, the maximum 8 as derived from tests of a week's production. It can be seen in Fig. 1 that the carbonyl index increases with decreasing taste scores, *i.e.* deteriorating oil quality. Assuming a linear relationship between the two characteristics, a regression coefficient of 0.85 was obtained

The method is suitable for the comparison of various refined and stored rape seed oil samples.

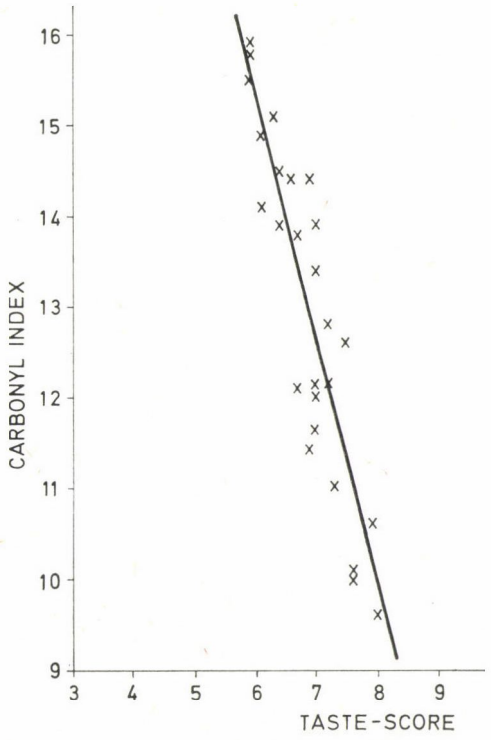

Fig. 1. Relationship between the sensory evaluation results (taste scores) and the carbonyl index

2.2. *Volatile carbonyl content*

The spectrophotometric volatile carbonyl index characterizes the amount of the low-molecular-weight volatile carbonyl compounds of the rape seed oil samples which can be removed by steam distillation.

The reproducibility of the test is shown in Table 2 where the results obtained with raw and refined rape seed oils are summarized. It can be seen that the relative standard deviation of the test is less than 5% with raw oil samples. Due to the extremely low absorbances fresh, refined samples cannot be compared by this method.

The volatile carbonyl index is eminently suitable for the characterization and comparison of rape seed oil samples stored for considerable lengths of time.

Gas chromatographic analysis, the other method used for the characterization of volatile carbonyl compounds, evaluates the volatiles removed by the carrier gas at the temperature of the evaporator block. Along with the carbonyls, other volatile compounds are also present in this chromatogram.

Table 2

The volatile carbonyl index of rape seed oil samples (four parallel determinations)

Rape seed oil sample	Volatile carbonyl index (average ± standard deviation)	Coefficient of variation (%)
No. 1 Raw	0.76 ± 0.01	± 1.1
No. 2 Raw	0.91 ± 0.04	± 4.2
No. 1 Refined	0.04 ± 0.01	±25.0
No. 2 Refined	0.07 ± 0.02	±28.6

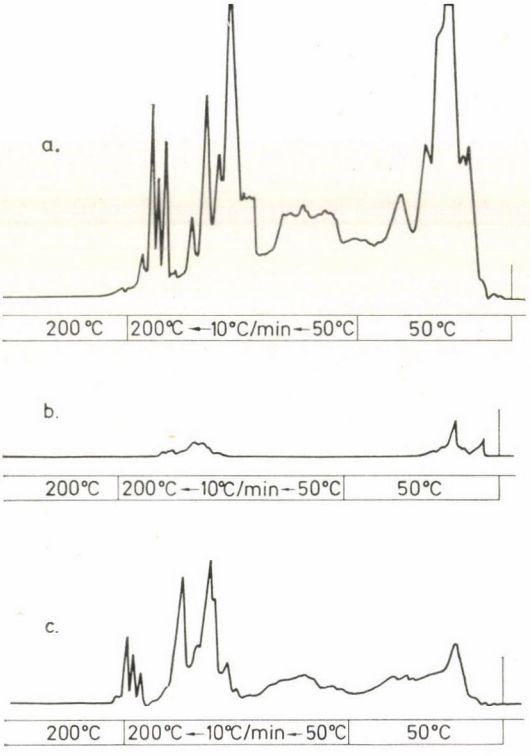

Fig. 2. Chromatograms of volatile compounds present in the rape seed oil samples. a) Raw rape seed oil; b) refined rape seed oil; c) refined rape seed oil stored for 9 months

The chromatograms of raw, fresh, refined and stored refined(over extended periods) rape seed oil samples are shown in Fig. 2. These fingerprint chromatograms are characteristic of the samples.

The volatile components cover the C_5–C_{15} n-paraffin retention time range in the 50–200 °C temperature range. Compounds with alkyl chains shorter than C_8 elute at 50 °C.

The gas-chromatographic method used is suitable for the comparison of raw and refined, as well as stored rape seed oil samples.

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ATTEMPTS TO UTILIZE WHEY FOR THE PRODUCTION OF YEAST PROTEIN. — PART. I. EFFECT OF WHEY CONCENTRATION OF AMMONIUM SULPHATE AND OF PHOSPHATE

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Whey can be used as a substrate for growing *Saccharomyces fragilis*, which from among the strains investigated previously, was the best cultured on whey medium.

The best lactose concentration of whey medium, giving the highest growth rate, was 2%. Lactose concentration higher than 2% had no significant effect on yeast propagation, but lactose concentration lower than 1% had a limiting effect on the growth rate of *S. fragilis*.

The addition of nitrogen and phosphorus salts to whey medium, using column and BIOFER fermentors, caused slight increases in growth rate, net dry weight and protein content of *S. fragilis*. (0.32 h^{-1} 5050 $\mu g\ cm^{-3}$ and 42.4 $\pm$ 0.13%; 0.35 h^{-1}, 6520 $\mu g\ cm^{-3}$ and 43.78 $\pm$ 0.23%, resp.)

Due to better aeration and agitation in the BIOFER system, the growth of *S. fragilis* was better in the BIOFER than in the column fermentor.

Whey can cause a water pollution problem if disposed of as a dairy waste. At present, 100 billion pounds of liquid whey are produced in the world annually (Vananuvat & Kinsella, 1975). It is well known that nine pounds of whey are produced on each pound of cheese. Whey can be used as a medium for yeast production suitable for supplementation of human and animal diets. Good yield of whey yeast has been obtained with *S. fragilis* (Enebo *et al.*, 1941). Yeasts grown on dairy products are higher in protein and amino nitrogen content than other yeasts; they are also rich in ascorbic acid, thiamin and riboflavin (Springer, 1949). Thus the propagation of yeast on whey may offer a means of obtaining an enriched feed supplement and at the same time reduces the pollution load of environment.

This paper deals with the establishment of suitable and economical lactose concentrations for growing *S. fragilis* in whey, and with the effect of and supplementation with 0.5% (w/v) each of $(NH_4)_2SO_4$ and of K_2HPO_4 on the propagation of *S. fragilis*. The latter was carried out on laboratory scale in a column fermentor and in a BIOFER fermentor.

1. Materials and methods

1.1. Organisms

A number of yeast strains were investigated for lactose utilization on mineral salt medium containing lactose as the carbon source (Table 1).

In previous experiments other authors found that *Saccharomyces fragilis* gave the highest yield (CHAPMAN, 1966; AMUNDSON, 1967; BECHTLE & CLAYTON, 1971; KNIGHT *et al.*, 1972; WASSERMAN, 1960a, b, 1961, 1962). Therefore in this study, *Saccharomyces fragilis* was mainly used. Yeast strains were maintained on malt agar slants, and transferred regularly to maintain viability.

Yeast strains *Brettanomyces clausenii*, NCYC 2; *Brettanomyces dublinensis*, NCYC 615, *Candida pseudotropicalis*, NCYC 152; *Saccharomyces fragilis*, NCYC 179; *Saccharomyces lactis*, NCYC 571; and *Saccharomyces lactis*, NACY 290 were obtained from the NATIONAL COLLECTION OF YEAST CULTURES (BREWING INDUSTRIES RESEARCH FOUNDATION, Nutfield, Surrey, England).

1.2. Media

1.2.1. Mineral medium. The yeast was cultivated in mineral medium of the composition described in Table 1.

Table 1

Composition of synthetic medium

$MgSO_4$	1.0 g
K_2HPO_4	1.4 g
$Na_2HPO_4 \cdot 12\,H_2O$	8.0 g
NaCl	10.0 g
$(NH_4)_2SO_4$	40.0 g
distilled water to make	10.0 l
lactose	4.5%
biotin	150 $\mu g\,l^{-1}$
yeast hydrolysate	10 $cm^3\,l^{-1}$
pH	4.5

1.2.2. Whey medium. Whey medium was prepared as follows. Spray-dried whey was reconstituted with distilled water to give lactose concentration of approximately 4.7% (w/v). Reconstituted whey was autoclaved for 15 min at a pressure of 122.58 kPa, and the precipitated proteins were removed by centrifugation. The clear supernatant was adjusted to pH = 7.0 with KOH (56.1 g l^{-1}) and again autoclaved. The precipitate was removed by centrifugation. The medium was adjusted to pH = 4.5 and autoclaved in flasks (LENORE & WASSERMAN, 1961). To prepare whey medium containing different lactose concentrations, different amounts of dried whey were reconstituted in water. Phosphorus and nitrogen sources were supplied in the form of K_2HPO_4 (0.5%) and $(NH_4)_2SO_4$ (0.5%).

1.2.3. Malt medium. Malt medium was prepared as follows: 70 g malt was dissolved in one l of distilled water, after autoclaving at 50.66 kPa for an hour, it was filtered, adjusted to pH = 4.5 and 5% sugar content, 3% agar-agar. Finally, the medium was autoclaved at 50.66 kPa for an hour.

1.3. Cultivation methods

1.3.1. Preparation of the inoculum. For inocula, yeasts were precultured on mineral agar in petri dishes for 24 h at 30 °C, then transferred to 150 cm^3 mineral medium placed in 500 cm^3 shaking flasks and cultured on a reciprocating shaker at 30 °C for 20 h. After centrifugation, the sedimented cells were resuspended in culture medium, to obtain an optical density of ca. 1.0 at 430 nm (ca. 640 μg cell dry matter per cm^3 medium).

1.3.2. Batch cultivation was carried out in a column fermentor and a laboratory-scale fermentor, type BIOFER (made by FÉMIPARI SZÖVETKEZET, Kecskemét).

1.3.2.1. Yeast cultivation in the column fermentor. We used a tube fermentor with automatic temperature control at 30 °C. The volume of fermentation broth was 500 cm^3. The culture was aerated with compressed air, sterilized by filtration. The aeration rate was 700 l h^{-1}. Foaming during yeast cultivation was prevented by addition of a synthetic antifoaming agent (STUCTOL, SCHILL WERKE, Hamburg). During cultivation, pH was maintained at 4.5 $\pm$ 0.5 by adding potassium hydroxide (1.0 *N*), or ammonium hydroxide (1.0 *N*). The volume of the medium was maintained at 500 cm^3. Evaporation losses were corrected by the addition of distilled water.

Cultivation was started by adding inoculum to the medium in an amount to reach a cell concentration of about 600–700 μg l^{-1}.

1.3.2.2. Yeast cultivation in a BIOFER*-type laboratory fermentor.* The BIOFER fermentor is a Hungarian made, highly instrumented and automated, 15-l capacity equipment for laboratory-scale work, with agitation and aeration devices, automatic temperature control and pH control. The authors found that addition of 1 cm^3 synthetic antifoam agent per liter before starting the experiment prevents the foaming during the whole period of fermentation*.

The speed of agitation during cultivation was varied as described in the results, but it started at 600 rpm. The culture was aerated with compressed air sterilized by filtration, the aeration rate was 200 l h^{-1} at the beginning and was varied during the fermentation time as described in the results.

* In preliminary experiments, difficulties were experienced with foaming, particularly when whey was used, while in synthetic media, there was no problem. By adding 1 cm^3 synthetic antifoam agent per 1 of medium before the start of fermentation, foaming could be eliminated. In case of highly foaming broth there was a great difference in pH values as measured in the circulation cuvette of the equipment and those of the probe.

Growth temperature was 30 °C and pH was 4.5 $\pm$ 0.05. The fermentation broth was inoculated with yeast suspension to a starting concentration of above 600 mg l^{-1}. Furthermore, the BIOFER instrument unit enables the measuring and registration of dissolved oxygen (DO), the redox potential (RP) and the CO_2 content of effluent gas.

1.4. Determination of cell growth

Growth of *S. fragilis* during cultivation was determined at every h as follows:

10 cm^3 of the culture broth were centrifuged, and the sedimented cells were washed with distilled water. The cells thus obtained were suspended

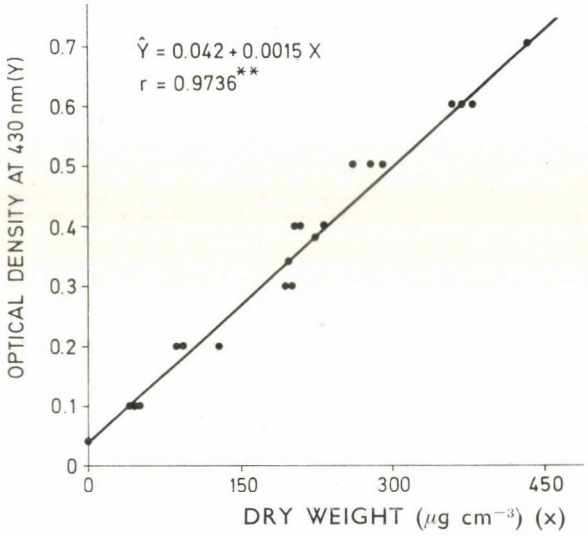

Fig. 1. The relation between optical density and dry weight of *Saccharomyces fragilis* NCYC 179

in 10 cm^3 of distilled water and the optical density of the suspension was measured at 430 nm. The dry cell content was determined from a calibration curve showing optical density as a function of dry cell weight (Fig. 1) (KNIGHT *et al.*, 1972; EDWARDS *et al.*, 1972; HIRAI *et al.*, 1972; BESTIC & ARNOLD, 1976).

1.5. Lactose determination

The supernatant was analysed for lactose concentration by the SOMOGYI method (1952).

1.6. Protein content of yeast

Batch cultivation was stopped after 9 h. Yeast cells were harvested by centrifugation. The cell paste was washed with distilled water and centrifuged. The washed cell paste was dried by an infrared lamp. Yeast protein content was determined by a *Kjel-Foss* automatic analyser Mod. 16210. (A/S Foss Electric, Denmark.)

The protein content of the samples was calculated on the basis of $N \times 6.25$.

1.7. Nucleic acids in yeast

These were determined by ultraviolet absorption according to Schmidt and Thannhauser (1945), Schneider (1945).

1.8. Alteration of main fermentation parameters

Dissolved oxygen, pH, redox potential and CO_2 data were registered automatically. Sugar content and cell density were measured every hour manually.

2. Results and discussion

2.1. Effect of whey (lactose) concentration

This experiment was carried out in a column fermentor. Media were prepared to contain 4 different lactose concentrations (4.7, 3.0, 2.0 and 1.0%) by reconstituting different amounts of dried whey in water. Each medium was supplemented with 0.5% K_2HPO_4 and 0.5% $(NH_4)_2SO_4$ (w/v) (Wasserman *et al.*, 1960a, b; Updegraff, 1971; Knight *et al.*, 1972) 150 $\mu g\ l^{-1}$ biotin.

Other cultivation conditions are described under *Materials and methods.*

The specific growth rate (h^{-1}) of *S. fragilis* decreased with decreasing lactose concentration (Table 2, Fig. 2).

The results show no significant difference in specific growth rate at lactose concentrations of 4.68, 3.08 and 2.10%. On the other hand, there is a highly significant difference between k values obtained at lactose concentration 1.05% and those at 2.10, 3.08 and 4.68%, resp.

According to *Monod* 's equation, the relation between lactose concentration (S) and specific growth rate (k)

$$k = k_{max} \frac{S}{k_s + S}$$

where k_{max} = maximum growth rate,
S = concentration of limiting nutrient giving highest growth rate,
k_s = concentration of nutrient at which $k = k_{max}/2$.

Table 2

The effect of lactose concentration on the specific growth rate

Initial lactose concentration (S) (%)	Specific growth rate (k) (h^{-1})	
	$\bar{x}$	$\pm t_{0.5} \cdot s_{\bar{x}}$
4.68	0.346	0.03
3.08	0.330	0.03
2.10	0.320	0.03
1.05	0.246	0.03

Least significant difference = 0.0439

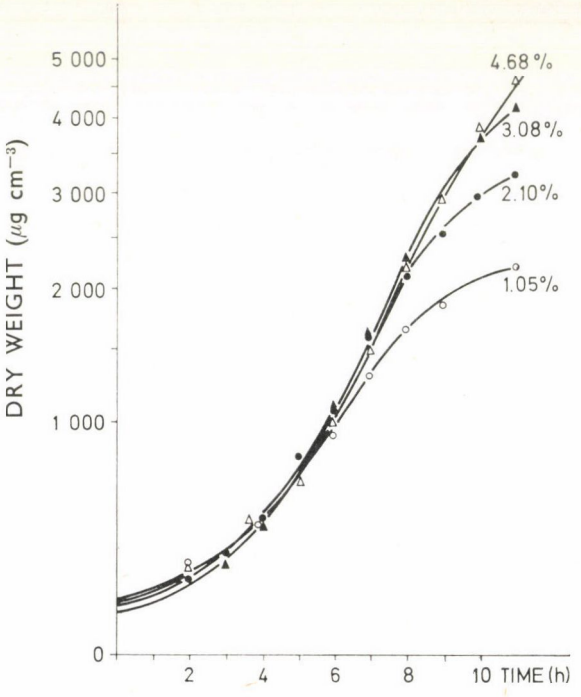

Fig. 2. Effect of whey lactose concentration on the growth of *Saccharomyces fragilis*. Symbols: —○—○— 1.05% lactose; —●—●— 2.10% lactose; —▲—▲— 3.08% lactose; —△—△— 4.68% lactose

Equally, by using the *Lineweaver* and *Burk* transformation and equation

$$\frac{1}{k} = \frac{k_s}{k_{max}} \cdot \frac{1}{S} + \frac{1}{k_{max}}$$

These relations are shown in Fig. 3

It is observed that, the best lactose concentration was 2%. A lactose concentration higher than 2% is not necessary and has no significant effect on the specific growth rate, but values lower than this, limit the growth rate.

The effect of the concentration of lactose in the medium is reflected in the protein content of the yeast. The results in Table 3 indicate that the crude protein in dry weight of the harvested yeast was 40–44%. This value was slightly decreased by decreasing the lactose content of the medium.

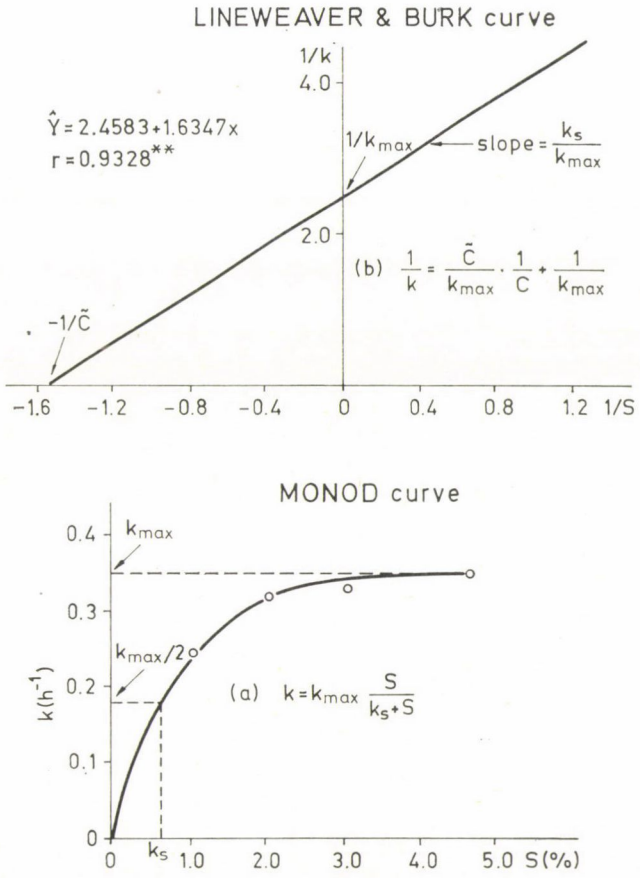

Fig. 3. Relationship between whey lactose concentration (S) and specific growth rate (k)

Table 3

The effect of lactose concentration on the protein content of the dry matter of S. fragilis

Substrate	Protein content (%) in the dry matter of harvested yeast			
	Crude protein (I)		Without RNA nitrogen (II)	
	$\bar{x}$	$\pm t_{0.5} \cdot S_{\bar{x}}$	$\bar{x}$	$\pm t_{0.5} \cdot S_{\bar{x}}$
Whey 4.7% lactose + N. + P.	43.78	1.01	32.81	0.46
Whey 3.0% lactose + N. + P.	42.07	1.13	31.88	0.46
Whey 2.0% lactose + N. + P.	41.58	1.13	33.50	0.46
Whey 1.0% lactose + N. + P.	39.40	1.30	32.25	0.46

N. = 0.5% $(NH_4)_2SO_4$
P. = 0.5% KH_2PO_4
Avarage of three series = $\bar{x}$
Least significant difference is 1.67 (I), and 0.66 (II), $(N - N_{RNA}) \cdot 6.25$ (II)

These results agree with those of HARJU and co-workers (1976), VANANUVAT and KINSELLA (1975) who found that the protein content of *S. fragilis* was between 40 and 50%.

However, it is an interesting fact that, after deducting the RNA nitrogen, the protein content was higher in the yeast grown on whey with 2% lactose.

2.2. Effect of added nitrogen and phosphorus salts

Two experiments were carried out in the BIOFER fermentor. The medium in the first was a medium with 2% lactose, but in the second one, the medium was supplemented with 0.5% each of $(NH_4)_2SO_4$ and K_2HPO_4 (w/v).

It was observed that the addition of these salts has caused a slight change in specific growth rate (0.32 *vs.* 0.35 h^{-1}) and there were slight differences in net dry weight after 9 h of cultivation (5050 and 6520 $\mu g\ cm^{-3}$, resp.) (Fig. 4).

From the preliminary studies on the growth of *S. fragilis* on whey, 4.7% lactose and modified whey with 0.5% from each $(NH_4)_2SO_4$and K_2HPO_4 (w/v) using a column fermentor we also found very slight differences in specific growth rate (0.35 and 0.37 h^{-1}, resp.), as well as little change in net dry weight after 9 h of cultivation.

In spite of the fact that there was no significant difference between the growth rates in the column and in the BIOFFR fermentor (Fig. 5), there was a highly significant difference in net dry weight. This result is due to the effect of better aeration and agitation in the BIOFER system.

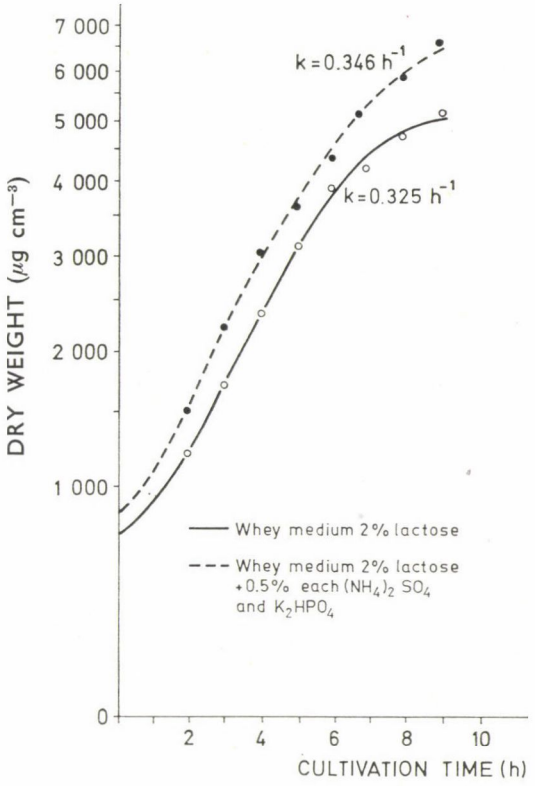

Fig. 4. Effect of supplementation of whey medium of 2% lactose with 0.5% each of $(NH_4)_2SO_4$ and K_2HPO_4 on the growth of *Saccharomyces fragilis* in a BIOFER fermentor. Symbols of curves: —○—○— $k = 0.325\ h^{-1}$; —●—●— $k = 0.346\ h^{-1}$

2.3. The pH value during fermentation in the BIOFER *equipment*

There is no significant difference in pH between the two experiments I and II (Figs. 6–7), but within the same experiment there was a difference in pH as measured in the probe and in the cuvette, presumably due to the effect of CO_2 (LENGYEL & NYIRI, 1965, 1966; NYIRI & LENGYEL, 1968).

2.4. CO_2 production during lactose utilization

If the dissolved oxygen concentration decreases below the critical range of DO, formation of CO_2 is inhibited. In our experiments, dissolved oxygen concentration of culture medium was kept in every case above the limiting level.

Adequate value of dissolved oxygen concentration will be guaranteed in two ways.

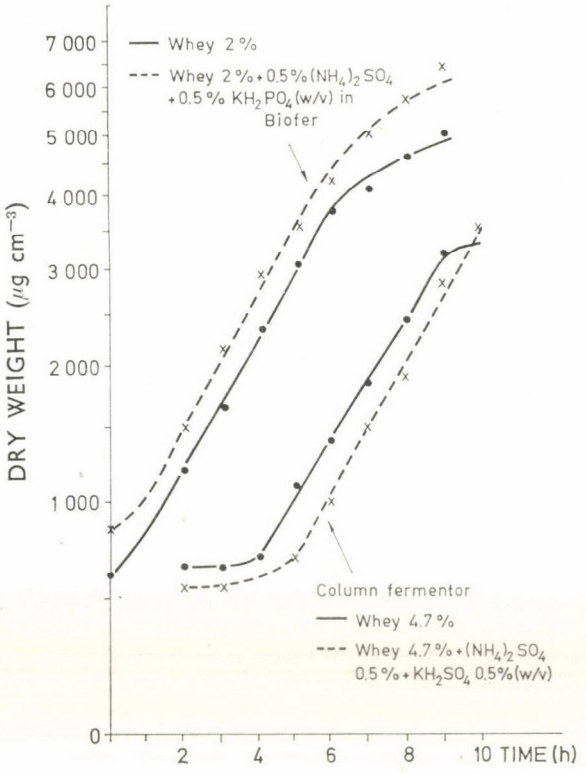

Fig. 5. Relationship between dry weight during growth in BIOFER fermentor. Symbols of curves: in BIOFER —·—·— whey 2%; —×—×— whey 2% + 5% $(NH_4)_2SO_4$ + 5% KH_2SO_4; in column fermentor: —·—·— whey 4.7%; —x—x— whey 4.7% + $(NH_4)_2SO_4$ 5% + KH_2SO_4 5%

— by speeding up the rotation of the mixer
— by using a higher rate of aeration.

CO_2 concentration of the air leaving the system is increased in case of speeding up agitation however, using a higher rate of aeration, causes the volume of the produced CO_2 to change. The CO_2 concentration of effluent gas and the volume of produced CO_2 in batch fermentation is mainly dependent on the growth phase of the cells. Both CO_2 values reach their maximum at the most intensive growth rate (Figs. 6—7).

Highest CO_2 production rates are registered at the 3rd–5th h of fermentation. At the same time, the maximum growth rate and lactose utilization was at the 3rd–5th h of fermentation. It appears to be more economical to carry out the fermentation at lower aeration rate which gives a higher CO_2 concentration in the effluent gas, but does not yet affect the rate of yeast production.

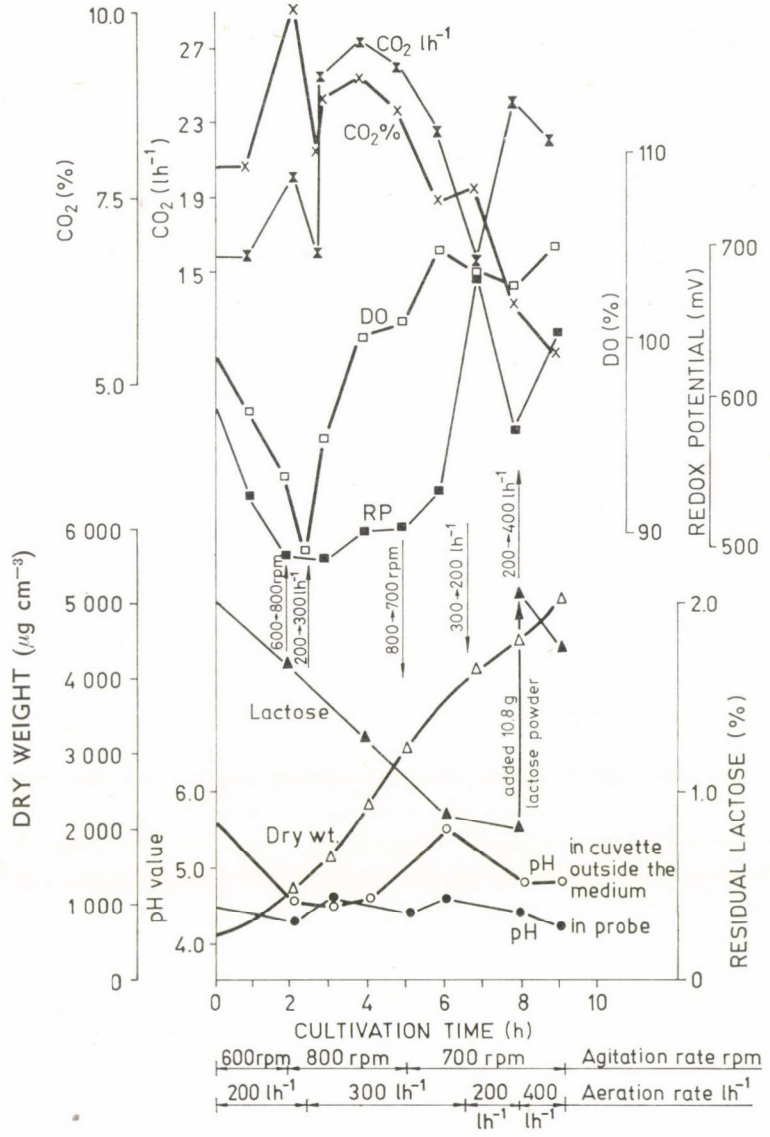

Fig. 6. Fermentation parameters during cultivation of *Saccharomyces fragilis* in whey medium of 2% lactose in BIOFER fermentor. Symbols of curves: —×—×— CO_2%; —x—x— CO_2 l h^{-1}; —□—□— dissolved oxygen; —■—■— redox potential; —△—△— dry weight; —▲—▲— residual lactose %; —○—○— pH in cuvette outside the medium; —●—●— pH in probe

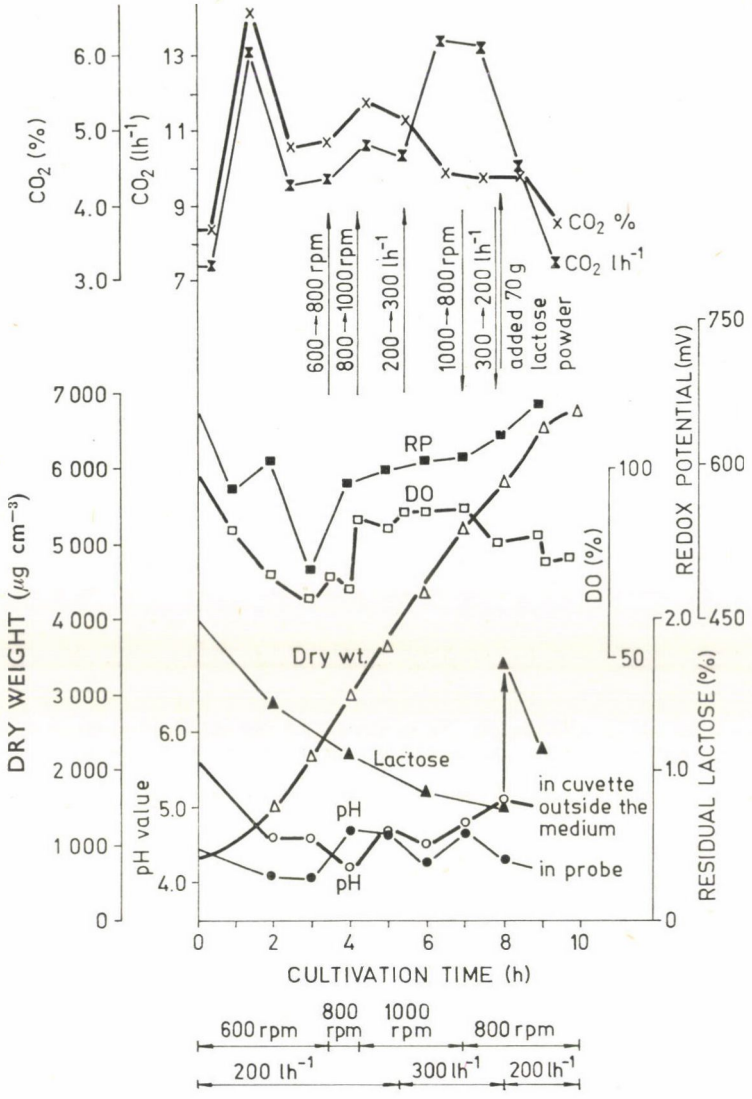

Fig. 7. Fermentation parameters during cultivation of *Saccharomyces fragilis* in whey medium of 2% lactose supplemented with 0.5% of each $(NH_4)_2SO_4$ and K_2HPO_4 in BIOFER fermentor. Symbols of curves: —×—×— CO_2 %; —x—x— CO_2 l h^{-1}; — — — dissolved oxygen; —■—■— redox potential; —△—△— dry weight; —▲—▲— residual lactose %; —○—○— pH in cuvette outside the medium; —●—●— pH in probe

However, at the estimated values, CO_2 had no stimulating, nor any inhibiting effect on the growth of yeast in this experiment because

– the inoculum was active and produced CO_2 for good germination rate,

– the CO_2 concentration in effluent gas in the laboratory fermentor was below the inhibitory concentration of CO_2.

2.5. Dissolved oxygen concentration during fermentation

DO depends on the rates of aeration and oxygen consumption, resp. The minimum value was between the 3rd–5th h (Figs. 6–7) in good agreement with the rate of CO_2 production.

2.6. Changes in redox protential

As well known from the literature, the change in RP in highly aerated culture liquids depends on the actual level of DO concentration. In the present study DO was always maintained at a concentration above the limiting value. Thus, RP correlated with oxygen concentration.

2.7. Changes in lactose content during fermentation

Consumption of lactose was relatively low during the first 3 h of cultivation. During this period the organism was adapting to the new environment. After the 3rd h, lactose consumption increased very rapidly. The consumption of lactose was very high and fairly constant after 8 h of cultivation at several agitation rates (Figs. 6–7). This result agrees with the findings of VANANUVAT and KINSELLA (1975). Lactose concentration in whey was reduced to 1.0–0.8% (in agreement with LANE's results, 1977). After adding pure lactose, a higher CO_2 production could again be observed, indicating that, below a lactose concentration of 0.8–1.0%, the rate of respiration is limited. Thus, it is suggested that there is a possibility for prolonging the production period of yeast.

The effects of the addition of nitrogen and phosphorus to whey are reflected in the protein content of the yeast. Thus, in yeast grown on a whey medium without added nitrogen and phosphorus, the protein content of the harvested yeast is slightly lower (42.4 ± 0.13) than that of the other yeasts grown on whey media containing 0.5% $(NH_4)_2SO_4$ and 0.5% K_2HPO_4 ($\bar{x} \pm s_{\bar{x}} = 43.78 \pm 0.23\%$). This phenomenon was observed in fermentations in columns and in the BIOFER apparatus alike. The nucleic acid content of harvested dry yeasts ranged from 6.3 to 10.6%.

3. Conclusions

Whey, a by-product of cheese processing, can be used as a substrate for the growth of *S. fragilis*. These studies were carried out in a column fermentor and in a BIOFER fermentor.

It was noticed that the specific growth rate (k) of *S. fragilis* decreased by decreasing the lactose concentration of the medium. The optimum lactose concentration which gives the highest growth rate was 2%. The protein content related to dry weight of the harvested yeast decreased by decreasing the lactose concentration of the medium.

The addition of nitrogen and phosphorus salts to whey media caused (a) a slight change in growth rate, and there are slight differences in net dry weight after 9 h of cultivation, (b) no significant difference between the growth rates in column fermentor and in BIOFER, but there were highly significant differences in net dry weight in case of the two types of fermentors. The protein content in the dry matter of harvested yeast was increased by the addition of nitrogen and phosphorus sources.

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STUDY OF VOLATILE SUBSTANCES PRODUCED DURING THE AUTOLYSIS OF CHAMPAGNE YEAST

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Intact and distintegrated Champagne Hautvillers yeast were added to tank-fermented as well as model sparkling wine and stored at different temperatures. Then, the changes in the composition of certain aroma substances were examined in the sparkling wine by gas chromatography. The concentrations of components of relatively high boiling point, *viz.*, of ethyl caproate, ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate, ethyl linoleate, *cis*-farnesol and *trans*-farnesol increased considerably. Raising of the temperature of seasoning enhanced the increase in the volatile components under study in the champagne. If sparkling wine had been aged in the presence of the same amount (expressed in yeast dry matter) of disintegrated yeast, the volatile substances would have reached 2 to 5 times as high concentrations in the sparkling wine as in the presence of intact yeast.

Sparkling wine aged with champagne yeast has better organoleptic properties than the tank-fermented yeast aged without added yeast. The better flavour is attributed to compounds released during the autolysis in the course of seasoning (FARRER, 1956). JACHINA and VOSTRIKOVA (1972) examined by thin-layer chromatography the terpene substances released during the autolysis of yeast. They found that the concentration of farnesol increased while that of geraniol and β-phenylethyl alcohol diminished in the course of the autolysis. During autolysis at 67 °C ,the concentration of β-ionone significantly increased. EDELÉNYI and co-workers (1975) studied the flavour and aroma substances in a base wine for sparkling wine making, two raw bulk-fermented sparkling wines and three bottle-fermented raw sparkling wines. They detected 21 components, of which 9 different alcohols were identified. Examining sparkling wines, produced by using different technologies, they found the most pronounced differences in the proportion of different components. EGOROV and co-workers (1975) examined ethyl oleate and other fatty acid esters as well as *cis*- and *trans*-farnesol quantitatively. They attribute the qualitative properties of sparkling wine to these components. RODOPULO and co-workers (1975) examined by gas chromatography the aroma substances in French and Soviet sparkling wines. In their opinion, the character of the sparkling wine is determined first of all by volatile components of relatively high boiling point. These authors attribute the characteristic sunflower scent to ethyl linolate, *cis*-farnesol and *trans*-farnesol. Gas chromatograms of

excellent French champagnes showed a number of peaks at the end of the curves. The authors suggest that the number of these peaks is in direct relation to the good quality of the sparkling wine. KORMAKOVA and DRBOGLAV (1976) examined the effects of preparation and seasoning on the quantities of different components (aldehydes, alcohols, esters *i.e.*) in the sparkling wine. They found that the concentrations of diacetyl, acetaldehyde, ethyl acetate and ethyl lactate increased on shaking up of the sparkling wine. In unshaken samples the substances of relatively high boiling point predominated among the examined volatile components.

1. Materials and methods

1.1. Microorganism

Champagne Hautvillers yeast maintained on slant malt agar was used throughout.

1.2. Fermentation of yeast

The fermentation of yeast has been described elsewhere (MOLNÁR *et al.*, 1980).

1.3. Preparation of yeast extract by disintegration of yeast cells

Tank-fermented sparkling wine and model sparkling wine were used in the experiments. Latter contained 12% (v/v) alcohol, 1 g l^{-1} tartaric acid, 2 g l^{-1} malic acid and 1 g l^{-1} citric acid dissolved in CO_2-saturated water. Samples from tank-fermented and model sparkling wine were stored in the presence of intact champagne yeast, 5.0 g yeast dry matter per l or disintegrated yeast 4.8 g yeast dry matter per l. The yeast was admixed at 0 °C to avoid CO_2 loss. Samples from each mixture were stored at 10, 20 and 30 °C for 90 days. In the course of storage, the sediments were shaken up at 20-day intervals.

1.4. Preparation of samples for examination of aroma substances

Champagne and model champagne samples were cooled to 0 °C and centrifuged at the same temperature at 3000 rpm for 10 min. The sedimented cells were removed. The supernatants were extracted in isopentane for 24 h, and each extract was concentrated to 1 ml in a *Vigreaux* column. Methyl caprylate, 490 mg l^{-1}, added to both the tank-fermented and model sparkling wine, served as the internal standard.

1.5. Gas-chromatographic analysis

The concentrated extract was analysed in a *Hewlett–Packard* type 5710 A gas chromatograph equipped with a flame ionization detector. The stainless column measuring 3 m × 1/8" was filled with *Chromosorb* W-AW (80–100 Mesh) containing 10% FFAP. The injecting block and the detectors operated at 200 °C and 300 °C, resp. Separation was performed by programmed heating including constant temperature at 80 °C for 4 min, followed by heating to 210 °C using a heating rate of 4 °C min^{-1}. At this level, the temperature remained constant until the end of the separation procedure. The quantitative determination and the identification of components were performed using the Laboratory Data Processing System of *Hewlett–Packard* 3352 B. The retention times of the components to be identified were determined by chromatographing pure standard substances, and identification of components was based on their retention time as related to that of the methyl caprylate used as an internal standard. The quantitative analysis was based on the areas related to the standard area. The apparatus calculated the actual concentrations from these quotients, taking into account the relative response factor.

2. Results and conclusions

Examining the effects of the autolysis of champagne yeast, we added intact or disintegrated champagne yeast to tank-fermented sparkling wine; the yeast dry-matter concentration was 5.0 g per l and 4.8 g per l, resp. Samples from these and from tank-fermented sparkling wine used as control were stored at 10, 20 and 30 °C for 90 days meanwhile the sediments were shaken up at 10-day intervals. After the end of the 90-day period, the following components were determined in each sample: ethyl caproate, ethyl caprylate, ethyl pelargonate, linalool, linalyl acetate, ethyl caprate, α-terpineol, α-phenyl ethyl acetate, ethyl laurate, β-ionone, ethyl myristate, ethyl palmitate, ethyl palmitoleate, *cis*-farnesol, *trans*-farnesol, ethyl stearate, ethyl oleate and ethyl linoleate.

The results, each representing the average for 3 samples, are shown in Figs. 1–4. The effects of the treatment on the concentrations of the volatile components presented in Tables 1–3 have been evaluated statistically (Table 4).

According to Tables 1–3, the tank-fermented sparkling wine to which neither intact nor disintegrated yeast had been added already contained the listed components, these being natural components of wine, especially of sparkling wine.

Storage of sparkling wine in the presence of intact yeast led to a slight increase in the quantities of components of relatively low boiling point, com-

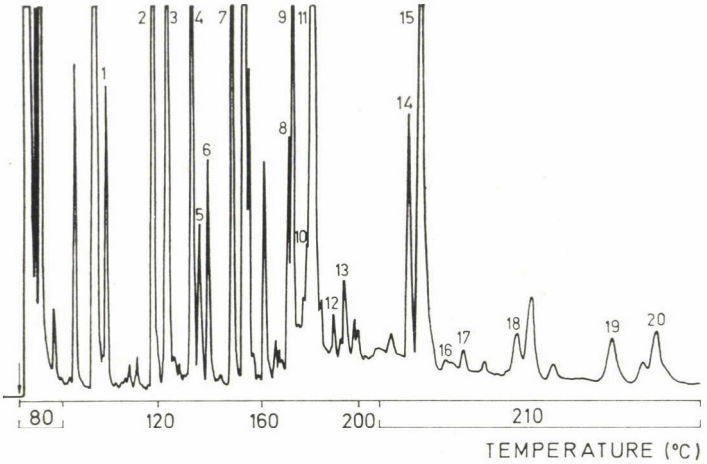

Fig. 1. Gas chromatogram of the volatile aroma fraction of sparkling wine stored with Ch. Hautvillers champagne yeast at 10 °C. Temperature of injector: 200 °C, temperature of detector: 300 °C. The temperature programme is presented in the Figure. *Peaks:* 1: ethyl caproate, 2: methyl caprylate, 3: ethyl caprylate, 4: ethyl pelargonate, 5: linalool, 6: linalyl acetate, 7: ethyl caprate, 8: α-terpineol, 9: β-phenylethyl acetate, 10: ethyl laurate, 11: β-phenylethyl alcohol, 12: β-ionone, 13: ethyl myristate, 14: ethyl palmitate, 15: ethyl palmitoleate, 16: *cis*-farnesol, 17: *trans*-farnesol, 18: ethyl stearate, 19: ethyl oleate, 20: ethyl linoleate

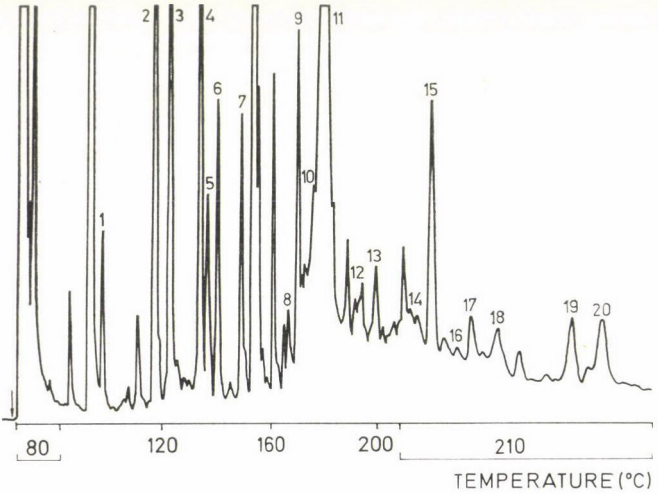

Fig. 2. Gas chromatogram of the volatile aroma fraction of sparkling wine stored on Ch. Hautvillers champagne yeast homogenate at 10 °C. For details of gas chromatography see Fig. 1. For explanation of peaks, see legend to Fig. 1

pared to the control values. The final concentrations of ethyl caproate, ethyl pelargonate, linalool, linalyl acetate, α-terpineol and phenylethyl acetate did not depend on the temperature of storage.

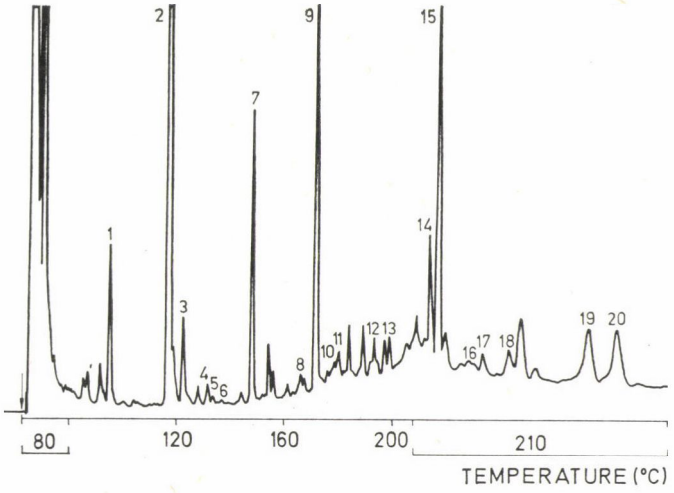

Fig. 3. Gas chromatogram of the volatile aroma fraction of model sparkling wine stored on Ch. Hautvillers champagne yeast at 10 °C. For details of chromatography, see Fig. 1. For explanation of peaks, see legend to Fig. 1

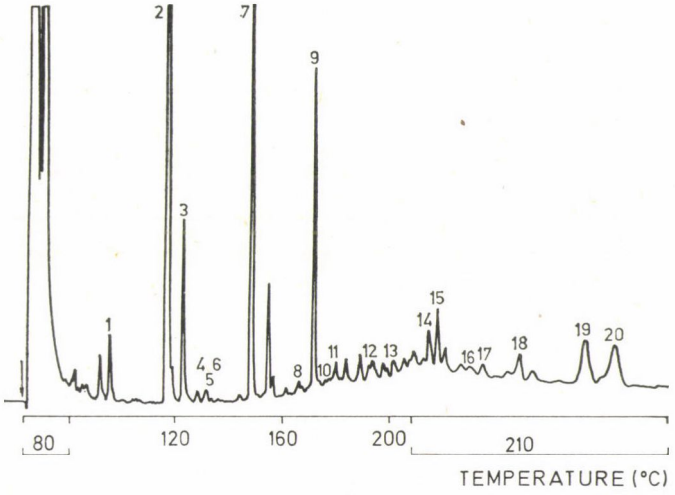

Fig. 4. Gas chromatogram of the volatile aroma fraction of model sparkling wine stored on Ch. Hautvillers champagne yeast homogenate at 10 °C. For details of gas chromatography, see Fig. 1. For explanation of peaks, see legend to Fig. 1

Ethyl laurate was not released from the autolysing yeast, and raising of the temperature of storage was followed by a decline in the final concentration of this component.

As a consequence of yeast autolysis the concentration of the following components increased in the sparkling wine: ethyl caprate, β-ionone, ethyl

Table 1

Effect of champagne yeast and its homogenate on the aroma substance composition of sparkling wine at 10 °C

Volatile aroma compounds	Tank-fermented sparkling wine			Model sparkling wine	
	Control	With yeast	With disintegrated yeast	With yeast	With disintegrated yeast
Ethyl caproate	145 ± 17	174 ± 22	302 ± 30	32 ± 6	165 ± 17
Ethyl caprylate	245 ± 33	342 ± 36	343 ± 36	84 ± 13	108 ± 13
Ethyl pelargonate	100 ± 16	135 ± 17	130 ± 17	16 ± 4	24 ± 4
Linalool	141 ± 23	168 ± 22	178 ± 27	25 ± 3	24 ± 4
Linalyl acetate	147 ± 17	179 ± 24	210 ± 29	36 ± 6	53 ± 7
Ethyl caprate	166 ± 12	345 ± 27	411 ± 38	225 ± 24	305 ± 34
α-terpineol	67 ± 15	74 ± 12	85 ± 13	8 ± 2	20 ± 4
β-phenylethyl acetate	172 ± 23	358 ± 39	685 ± 47	159 ± 20	517 ± 54
Ethyl laurate	178 ± 23	172 ± 21	172 ± 22	2	2
β-ionone	20 ± 4	26 ± 8	64 ± 11	9 ± 2	48 ± 7
Ethyl myristate	18 ± 4	27 ± 4	55 ± 9	7 ± 2	39 ± 6
Ethyl palmitate	17 ± 3	48 ± 10	236 ± 24	25 ± 4	223 ± 25
Ethyl palmitoleate	120 ± 17	147 ± 17	413 ± 41	16 ± 3	188 ± 29
Cis-farnesol	24 ± 3	36 ± 9	61 ± 11	14 ± 2	35 ± 5
Trans-farnesol	73 ± 17	120 ± 14	217 ± 25	54 ± 10	152 ± 17
Ethyl stearate	39 ± 10	41 ± 6	68 ± 11	15 ± 3	60 ± 8
Ethyl oleate	75 ± 12	122 ± 13	282 ± 29	45 ± 9	185 ± 21
Ethyl linoleate	91 ± 16	120 ± 12	257 ± 29	52 ± 29	174 ± 16

Mean concentrations and standard deviations (s) are presented in $\mu g\ cm^{-3}$

myristate, ethyl palmitate, ethyl palmitoleate, *cis*-farnesol, *trans*-farnesol, ethyl stearate, ethyl oleate and ethyl linoleate. The concentrations of these components directly related to the temperature of storage. The increase in, and the heat dependence of, the final concentrations of ethyl caproate, ethyl palmitate and ethyl stearate were especially striking: ethyl caproate concentration increased up to 345–410 $\mu g\ l^{-1}$, depending on the temperature,compared to the 120 $\mu g\ l^{-1}$ concentration in the tank sparkling wine. The respective values for ethyl palmitate were 236—274 $\mu g\ l^{-1}$ and 16—18 $\mu g\ l^{-1}$, for ethyl stearate 68–117 $\mu g\ l^{-1}$ and 16–19 $\mu g\ l^{-1}$.

When the tank-fermented sparkling wine was stored on disintegrated yeast, essentially similar changes were observed. The concentrations of ethyl caproate, ethyl caprylate, ethyl pelargonate, linalool, linalyl acetate, α-terpineol and β-phenylethyl acetate were 1.2 to 3 times the respective concentrations

Table 2

Effect of champagne yeast and its homogenate on the aroma substance composition of sparkling wine at 20 °C

Volatile aroma compounds	Tank-fermented sparkling wine			Model sparkling wine	
	Control	With yeast	With disintegrated yeast	With yeast	With disintegrated yeast
Ethyl caproate	150 ± 17	184 ± 21	314 ± 34	35 ± 5	170 ± 19
Ethyl caprylate	238 ± 35	335 ± 35	339 ± 36	80 ± 10	115 ± 14
Ethyl pelargonate	115 ± 15	146 ± 18	135 ± 16	18 ± 4	25 ± 4
Linalool	147 ± 17	172 ± 18	180 ± 22	26 ± 5	24 ± 5
Linalyl acetate	158 ± 18	182 ± 19	214 ± 23	34 ± 6	54 ± 6
Ethyl caprate	130 ± 15	374 ± 36	462 ± 45	250 ± 24	335 ± 35
α-terpineol	64 ± 8	73 ± 9	78 ± 9	15 ± 3	20 ± 4
β-phenylethyl acetate	188 ± 22	380 ± 37	696 ± 64	160 ± 19	524 ± 49
Ethyl laurate	120 ± 4	128 ± 15	124 ± 14	2	2
β-ionone	17 ± 4	36 ± 8	78 ± 10	14 ± 4	61 ± 8
Ethyl myristate	16 ± 4	38 ± 8	71 ± 9	19 ± 5	54 ± 6
Ethyl palmitate	16 ± 4	59 ± 10	263 ± 26	40 ± 7	245 ± 27
Ethyl palmitoleate	124 ± 14	175 ± 19	443 ± 42	48 ± 8	324 ± 34
Cis-farnesol	23 ± 5	47 ± 7	70 ± 9	26 ± 4	53 ± 7
Trans-farnesol	70 ± 8	144 ± 17	235 ± 22	77 ± 8	185 ± 22
Ethyl stearate	16 ± 4	48 ± 7	104 ± 13	20 ± 4	86 ± 10
Ethyl oleate	91 ± 11	160 ± 19	305 ± 32	90 ± 11	217 ± 23
Ethyl linoleate	90 ± 10	182 ± 20	291 ± 31	112 ± 13	209 ± 20

Mean concentrations and standard deviations (s) are presented in $\mu g\ cm^{-3}$

reached in the tank-fermented sparkling wine. The rate of concentration increase was independent of temperature and comparable to the values obtained from the similar experiments with intact yeast.

The concentration of ethyl caproate, β-ionone, ethyl myristate, ethyl palmitate, ethyl palmitoleate, *cis*-farnesol, *trans*-farnesol, ethyl stearate, ethyl oleate and ethyl linoleate considerably increased in the sparkling wine samples and directly related to the temperature of storage. The concentration increase was the most pronounced with ethyl caproate, ethyl palmitate and ethyl stearate. The final concentration of these esters was 410–490, 236 and 68–117 $\mu g\ l^{-1}$, resp., depending on the temperature.

Experiments with model sparkling wine showed that, from the disintegrated yeast, 2 to 5 times as much volatile substance was released as from the intact yeast. The differences were more pronounced with the compounds

Table 3

Effect of champagne yeast and its homogenate on the aroma substance composition of sparkling wine at 30 °C

Volatile aroma compounds	Tank-fermented sparkling wine			Model sparkling wine	
	Control	With yeast	With disintegrated yeast	With yeast	With disintegrated yeast
Ethyl caproate	148 ± 17	171 ± 22	308 ± 32	31 ± 5	173 ± 18
Ethyl caprylate	254 ± 27	353 ± 33	349 ± 37	92 ± 12	114 ± 13
Ethyl pelargonate	117 ± 14	140 ± 18	142 ± 15	27 ± 4	28 ± 4
Linalool	148 ± 17	166 ± 19	175 ± 21	14 ± 3	25 ± 5
Linalyl acetate	156 ± 17	170 ± 17	210 ± 23	19 ± 4	58 ± 6
Ethyl caprate	118 ± 11	410 ± 44	491 ± 47	290 ± 30	364 ± 34
α-terpineol	70 ± 10	81 ± 11	92 ± 11	8 ± 3	24 ± 4
β-phenylethyl acetate	175 ± 20	366 ± 31	700 ± 59	212 ± 26	530 ± 47
Ethyl laurate	70 ± 11	67 ± 8	71 ± 9	2	2
β-ionone	15 ± 4	45 ± 6	86 ± 10	31 ± 6	72 ± 8
Ethyl myristate	15 ± 4	44 ± 6	78 ± 9	26 ± 4	61 ± 7
Ethyl palmitate	18 ± 5	67 ± 8	272 ± 29	48 ± 6	260 ± 27
Ethyl palmitoleate	117 ± 12	200 ± 25	460 ± 43	74 ± 8	337 ± 32
Cis-farnesol	24 ± 6	56 ± 8	87 ± 10	36 ± 5	65 ± 6
Trans-farnesol	71 ± 8	158 ± 18	264 ± 28	93 ± 12	200 ± 21
Ethyl stearate	18 ± 4	56 ± 8	117 ± 10	35 ± 6	97 ± 12
Ethyl oleate	87 ± 10	210 ± 23	320 ± 31	140 ± 15	230 ± 24
Ethyl linoleate	84 ± 10	235 ± 24	302 ± 28	170 ± 18	221 ± 24

Mean concentrations and standard deviations (s) are presented in $\mu g\ cm^{-3}$

of high boiling point than with those of low boiling point. The model sparkling wine samples stored on intact yeast contained 25–48 $\mu g\ l^{-1}$ of ethyl palmitate, 16–74 $\mu g\ l^{-1}$ of ethyl palmitoleate, 15–35 $\mu g\ l^{-1}$ of ethyl stearate, 45–140 $\mu g\ l^{-1}$ of ethyl oleate and 62–170 $\mu g\ l^{-1}$ of ethyl linoleate. The respective values for samples stored on disintegrated yeast were as follows: 233–260, 288–337, 60–97, 183–230 and 174–221 $\mu g\ l^{-1}$. In conclusion, greater amounts of aroma components diffused into the sparkling wine from the disintegrated than from the intact yeast.

Organoleptically, we found the sample stored on disintegrated yeast at 10°C the most favourable. If the temperature was higher the organoleptic properties of the sparkling wine were the less favourable the higher the temperature was. The relation was similar when the properties of the model sparkling wines were compared with those of the ones stored at the same temperature.

Table 4

Effect of storage with champagne yeast or disintegrated champagne yeast on the averages of the volatile compounds studied

Treatments	$S_a - X_b$	SQ_a	SQ_b	s_d	t P_{Table}
Tank-fermented sparkling wine at 10 °C					
Control *vs.* yeast	47	194,516	71,049	29	$1.62 < P_{5\%} = 2.12$
Control *vs.* disintegrated yeast	132	445,298	71,049	41	$3.21 < P_{0.1\%} = 4.02$
Disintegrated yeast *vs.* yeast	85	445,298	194,516	45	$1.88 < P_{5\%} = 1.12$
Model sparkling wine					
Disintegrated yeast *vs.* yeast	89	306,368	57,587	34	$2.61 < P_{1\%} = 2.92$
Tank-fermented sparkling wine at 20 °C					
Control *vs.* yeast	61	201,536	732,984	30	$2.03 < P_{5\%} = 2.12$
Control *vs.* disintegrated yeast	146	477,635	73,284	42	$3.47 < P_{0.1\%} = 4.02$
Disintegrated yeast *vs.* yeast	85	477,635	201,536	47	$1.80 < P_{5\%} = 2.12$
Model sparkling wine at 20 °C					
Disintegrated yeast *vs.* yeast	91	334,384	67,089	36	$2.52 < P_{1\%} = 2.92$
Tank-fermented sparkling wine at 30 °C					
Control *vs.* yeast	72	223,688	74,225	31	$2.32 < P_{1\%} = 2.92$
Control *vs.* disintegrated yeast	157	502,862	74,225	43	$3.65 < P_{0.1\%} = 4.02$
Disintegrated yeast *vs.* yeast	85	502,862	223,688	48	$1.77 < P_{5\%} = 2.12$
Model sparkling wine at 30 °C					
Disintegrated yeast *vs.* yeast	83	352,382	107,959	38	$2.18 < P_{1\%} = 2.92$

No. of components studied: n = 18

We can state that during tank fermentation in the presence of Champagne Hautvillers yeast, the concentrations of the substances under study increased in the sparkling wine to a higher degree than in the sparkling wine stored without yeast.

As to the effect of temperature of storage, the compounds can be divided into the following three groups.

The increase in concentration of certain components, mainly those of relatively low boiling point is, practically, independent of the temperature. The ethyl laurate concentration even declined in samples kept at high temperature.

The concentrations of the components of high boiling point consistently increased with rising temperature.

Among the terpene-type compounds, linalool, linalyl acetate and α-terpineol showed slight concentration rises during storage on yeast, either intact or disintegrated. On the other hand, the concentrations of β-ionone, *cis*-farnesol, and *trans*-farnesol, the three characteristic products of yeast autolysis, increased considerably.

Ethyl myristate, ethyl palmitate, ethyl stearate, ethyl oleate and ethyl linoleate can also be regarded as components characteristic of the process of yeast autolysis.

Comparing the concentrations reached in the sparkling wine stored on intact yeast with those reached on disintegrated yeast (the dry matter contents were nearly the same), we found that from the disintegrated yeast 2 to 8 times as much of the components was released as from the intact yeast. The difference was especially pronounced in the case of ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate and ethyl linoleate. On the other hand, the dependence on temperature of storage was less pronounced with disintegrated yeast than with the intact one.

In summary, the amount of the volatile components studied in sparkling wine increased on seasoning either with intact or with disintegrated yeast. A greater amount of volatile components was produced by autolysis with disintegrated yeast, characterized mainly by the presence of aliphatic monocarbonic acid ethyl esters of higher carbon number.

In conclusion, the presence of great amounts of yeast in the seasoning of sparkling wine, and the consequent autolysis at 20–30 °C, is not always favourable as regards the organoleptic properties of sparkling wine.

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EFFECT OF GAMMA IRRADIATION ON THE CYTOKININS OF MUSHROOMS

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Freshly harvested mushrooms were irradiated (0, 1.0 and 2.5 kGy) and stored for 8 days at 14 ± 2 °C. The stored samples were studied for cytokinin content. The changes in the cytokinin activity were observed to be quite high especially during the first 4 days of storage. The lamella contained the highest concentration of cytokinin followed by stipe and pileus. Lamella exhibited highest concentration on 0 day while stipe and pileus showed highest concentration on 1 day with progressive decrease during the subsequent storage in all morphological parts. Irradiation strongly inhibited the cytokinin activity and 2.5 kGy had stronger inhibitory effect than 1.0 kGy.

Ripening and aging of fruit and vegetable are determined by the interplay of various hormone groups like auxins, gibberellines, abscissic acid and cytokinins (GERTMAN & FUCHS, 1972; THOMAS & ISENBERG, 1972). Cytokinins are involved in all phases of plant development *i.e.* from seed germination to plant aging and senescence. Moreover, these compounds have special stimulatory functions in cell division and callus growth in plant tissue culture (HEIDE, 1972). Levels of cell division factors are usually high in young developing fruits and seeds. MILLER (1967) reported that cytokinin levels rose during the early development of corn kernels and then dropped again as the fruit matured. Similarly, embryo and young fruits have been found to be the commonest sources of cytokinins in plant extracts (STEWARD & SHANTZ, 1956).

Many of the responses in plants elicited by ionizing radiation have been attributed to the destruction of growth hormones (DESROSIER & ROSENSTOCK, 1960). The present investigations have, therefoie, been undertaken to study the growth inhibiting effects of irradiation in relation to the cytokinin activity of mushrooms.

1. Material and methods

1.1. Material

Agaricus bisporus (L.) variety of mushrooms was used in the present investigations. The raw material for the examination was procured from the MUSHROOM PRODUCTION UNIT, DUNA AGRICULTURAL COOPERATIVE, Budapest. The fresh mushrooms were picked at the stretched-veil stage with

closed caps having a diameter of 3–5 cm. The fruit bodies were sorted for uniform shape and size, trimmed from the base and the soil was brushed off.

1.2. Radiation treatment

Radiation treatment of mushrooms was carried out in the ^{60}Co panoramic source of 3.07 PBq activity at the experimental plant of the INSTITUTE OF ISOTOPES of the HUNGARIAN ACADEMY OF SCIENCES. The samples were irradiated at the optimum dose rate of 1.0 and 2.5 kGy h^{-1}. Irradiation doses of 1.0 and 2.0 and 2.5 kGy were applied in air at 14 °C. One third of the mushrooms was used as non-irradiated control.

1.3. Storage conditions

Irradiated and unirradiated mushrooms were stored at 14 $\pm$ 2 °C and 80–90% relative humidity. The stored samples were examined for cytokinin activity after 0, 1, 2, 4, 6 and 8 days.

1.4. Isolation of cytokinin

The extraction of cytokinin was carried out by the method described by LETHAM and WILLIAMS (1969). Pileus, stipe and lamella portions of mushroom tissue (40 g) were blended separately for 5 minutes with 80% ethanol, three times the quantity of the sample. The homogenate was kept in a refrigerator overnight, and then filtered. The filtrate was evaporated under vacuum at 40 °C and set at pH 3.0. The concentrate was shaken with a 4-fold amount of ether. The ether phase, being cytokinin-inactive, was not used. The aqueous phase was set at pH 6.5, and then shaken with 4-fold volume of n-butanol saturated with water. The butanol phase was evaporated under vacuum (40 °C) and then purified.

1.5. Cytokinin separation and purification

Polyclar AT (GAF), an insoluble form of PVP (Polyvinylpyrrolidone), forms insoluble complexes with phenol, which may thus be removed from plant extract.

Polyclar AT (PVP) was washed with distilled water and phosphate buffer of 6.4 pH. The extracted, dried cytokinins were dissolved in 3–4 cm^3 of distilled water, filtered and applied to the prepared column and then eluted with further phosphate buffer of pH 6.4. The column flow rate was 36 cm^3 h^{-1}. The cytokinin elution profile was obtained by monitoring the transmittance of ultraviolet light at 254 nm through the eluate using an LKB *Uvicord* II absorbance meter (BIDDINGTON & THOMAS, 1973a).

1.6. Amaranthus betacyanin bioassay for the rapid determination of cytokinin

The *Amaranthus* betacyanin assay is based on the cytokinin-induced formation of the betacyanin in the cotyledons and hypocotyls of *Amaranthus* seedlings incubated in darkness in the presence of tyrosine.

Seeds of *Amaranthus paniculatus* (pigmitorch) (VETŐMAG CO. Budapest) were allowed to germinate in darkness at 25 °C for 96 h. Ten seedlings, without seed coats, were put in a small plastic box which contained 3.3 cm^3 of phosphate buffer, 2.2 cm^3 of tyrosine (1 mg cm^{-3}) and cytokinin fractions to be assayed. The boxes were incubated at 298 °K (25 °C) for 18 h in the dark after which the explants were removed and placed in 3 cm^3 of distilled water. Betacyanin was extracted by means of two or three cycles of freezing and thawing and their quantity determined by calculating the difference between optical densities at 542 and 620 nm (BIDDINGTON & THOMAS, 1973b).

2. Results

The tissue extracts of the mushroom's pileus, stipe and lamella portions were subjected separately for the collection of cytokinin fractions in an LKB apparatus. About 30 fractions in each case obtained were tested by the *Amaranthus* betacyanin biotest. Elution profiles of different mushroom portions are illustrated in Fig. 1.

The results indicated that no activity was shown by the first five fractions which were collected from the pileus, stipe and lamella portions of the tissue.

2.1. Effect of storage

Several factors contribute to the postharvest quality of mushrooms. Among these are growth (stipe elongation and pileus opening), discoloration and changes in texture.

Cytokinins are present in mushrooms. Since little is known of the postharvest behaviour of mushrooms, we investigated the effect of changes in cytokinin activity during storage of *Agaricus bisporus*.

It was found that prominent changes in the cytokinin activity had occurred during the storage period of 8 days. No activity was observed in any of the investigated portions after 2 days of storage in samples irradiated at 1.0 kGy dose. Similarly no cytokinin effects were detected in the samples irradiated at 2.5 kGy dose by the applied bioassay method on or after 1 day of storage, except for the stipe portion which showed negligible activity on the 1st day only.

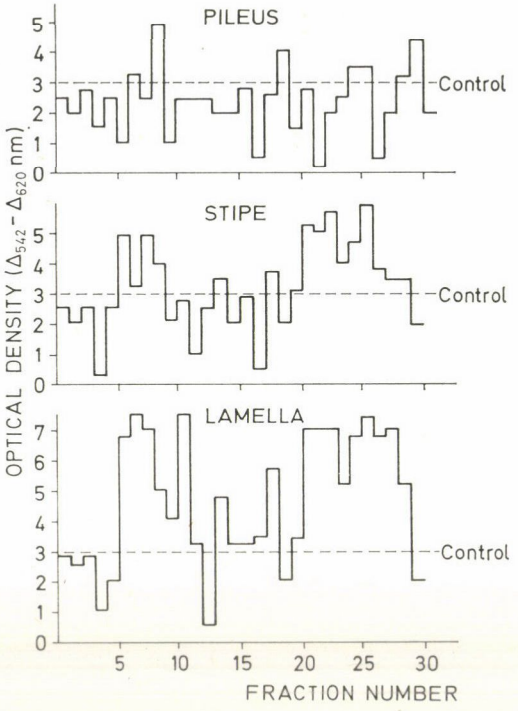

Fig. 1. Betacyanin production in the seedlings of *Amaranthus paniculatus* in the presence of the extracts of fresh mushroom

Less cytokinin activity was exhibited by the extracts of pileus and stipe portions of fresh mushroom at 0 day than after 1 day the difference is significant ($P < 0.01$). Highest activity was noted in these portions on 1 day storage with a consistent and significant ($P < 0.01$) decreasing trend during further storage. It was also observed that, in the fresh samples, higher (about 2 times) cytokinin activities were shown by the stipe than by the pileus of the mushroom, which increased or decreased similarly till the 2nd day after which the activity in stipe diminished quicker than in the pileus portion. The lamella of mushrooms which were freshly harvested showed an activity trend different from that of the pileus or of the stipe portions. It was noted that on 0 day the lamella portions had highest values which decreased consistently as a function of storage time. Further, it was also observed that, on 0 day, the lamella portion had about 3 and 6 times higher activities than the stipe and pileus portions, resp. The decrease of activity in samples stored for more than one day was similar as was observed in pileus and stipe portions.

It was noted that the stipe and pileus portions of mushrooms exhibited low cytokinin activities on 0 day and higher activities on the 1st day, decreasing later on progressively. This is in accordance with the findings of STEWARD

and SHANTZ (1956) who reported that embryo and young fruits are the commonest rich sources of cytokinins in plant extracts, and with the article by MILLER (1967) who found that cytokinin levels rose during the early developments of corn kernels and then dropped again as the fruits matured. KOVÁCS and VÖRÖS (1975) reported that the cytokinin content of unripe tomatoes was higher than that of the ripe tomatoes, which decreased with the advancement of ripening. In the present experiment it was observed that lamella portions of mushroom had highest concentration of cytokinin which also diminished in function of storage time. This can be positively compared with the finding of BURNETT (1968) who reported that the centre of growth lies just beneath the pileus. KOVÁCS (1978) showed that the gill was a rich source of cytokinin in mushrooms and the quantity decreased with storage time. As the cytokinin activity decreased on 1 day storage in lamella fractions while it increased in the pileus and stipe portions, it is expected that the cytokinins might have moved from one part of the plant to another.

2.2. Effect of irradiation

It was observed that there was no immediate effect of irradiation on the cytokinin activity of fresh pileus, stipe and lamella tissues of mushrooms, as no statistical difference in any of the 0, 0.1 and 2.5 kGy irradiation treatments could be demonstrated. Many of the cytokinin fractions obtained from fresh, 1.0 and 2.5 kGy treated sample extracts showed activities which nearly equalled that of unirradiated (0 kGy) samples. It was found that weak activity was exhibited by the extracts prepared from mushrooms irradiated at 1.0 kGy treatments after 0 day of storage in all the portions. Radiation dose of 2.5 kGy had highly significant effect ($P < 0.01$) on the inhibition of cytokinin activity which was evident from the fact that all the investigated samples exhibited no activity during their storage after 0 day, except that of stipe which gave a value of 32 ng zeatin g kg^{-1} sample on the 1st day with zero activity during further storage. KOVÁCS and VÖRÖS (1975) also observed the suppressing effect of irradiation on cytokinin in tomatoes during their storage for 8 days. In our experiments, the radiation treatments of 1.0 and 2.5 kGy differed significantly ($P < 0.01$), the latter producing more strongly suppressing effects than the 1.0 kGy dose of irradiation.

3. Conclusions

In the present investigation it was observed that the lamella of fresh mushroom had higher concentrations of cytokinins than the pileus and stipe portions. Cytokinin activity decreased in the lamella portion as a function

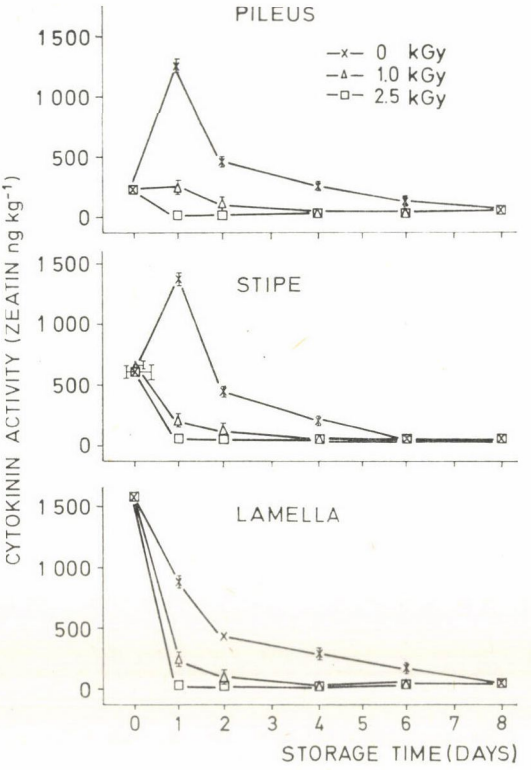

Fig. 2. Effect of irradiation on the cytokinin activity of mushrooms as a function of storage time [Temp.: 287 K (14 ± 2 °C), R. H.: 80–90%]

of storage time, while it increased, on storage, in the pileus and stipe portion in the beginning (1 day) and then diminished as a function of storage time. From the increasing and decreasing trends of cytokinin activity in different investigated portions, it can be suspected that the cytokinins have moved from one part to another, and were utilized for the ripening processes in mushrooms.

It was noted that irradiation doses of 1.0 and 2.5 kGy significantly ($P < 0.1$) inhibited the cytokinin activity in all the investigated portions of mushrooms. The effect of 2.5 kGy treatment was stronger than 1.0 kGy treatment. Since the high cytokinin activity is usually associated with cell division, it can be suspected that the energies absorbed by the mushrooms due to ionizing treatments were dissipated preferentially, by direct and indirect hit mechanisms, in the destruction of this growth hormone. Further work seems to be necessary to have a clear idea of the situation.

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SPECTRAL TRANSMITTANCE CHARACTERISTICS OF PEACHES, APRICOTS, PEARS, PLUMS, STRAWBERRIES, TOMATOES AND PEPPERS

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Colour and ripeness evaluation of some fruits and vegetables by instrumental measurement of their light transmittance is discussed. Spectral characteristics of whole fruits in the visible and near infra-red range were investigated. Transmittance at various pairs of wavelengths was correlated with data on the colour, ripeness and suitability for fresh consumption or canning. It was found that (a) the correlation coefficient between the difference in optical density at two wavelengths and the sensory colour and ripeness evaluation lies between 0.812 and 0.990; the wavelengths are in the range of 550–800 nm, (b) the light transmittance ratio for fruit from green to ripe varies between 5.4 for pears and $219 \cdot 10^3$ for tomatoes, (c) transmittance measurement of whole fruits at two wavelengths allows quick colour and ripeness evaluation, as well as automatic sorting into several fractions with the Bulgarian automatic colour sorter $ASC_{I/II}$.

Light transmittance measurement of whole (uncut) fruits allows quick quality evaluation in view of their further utilization for fresh consumption, canning, freezing, *etc.*

This method was used first for the non-destructive measurement of the internal tomato colour (BIRTH *et al.*, 1957; NORRIS, 1956) and later for the non-destructive determination of the stage of maturation, presence of diseases, pigment quantity and other characteristics of peaches, apples, oranges, plums, cherries, potatoes, *etc.* (GRÜNEWALD, 1974; KRIVOSHIEV, 1974; KRIVOSHIEV *et al.*, 1977; NEOTEC INSTRUMENTS, 1974; ROSENTHAL and WEBSTER, 1973; SIDWELL, 1961; USDA, 1965; WATADA, 1976).

The results reported here are the outcome of research work conducted during several years at the CANNING RESEARCH INSTITUTE, Plovdiv in order to clarify the possibilities for instrumental quality evaluation of major agricultural products and their automatic sorting with the Bulgarian automatic colour sorter ASC 1/11 (KRIVOSHIEV, 1974; KRIVOSHIEV *et al.*, 1977).

1. Materials and methods

The tests were carried out with sound fruits of regular form in various stages of maturation. The investigation included peaches of the *Fortuna*, *Baby Gold 5*, *Elberta*, *Halle* and *Corona* varieties; *Magyar Boys* apricots;

Bartlett, *Bosc* and *Passe Crassane* pears; plums and greengages; *Gorella* strawberries (frozen); tomatoes of the *Extase*, *Panase* C_3F, *Virase*, *VF 198*, *VF 316*, *VF 145*, *Chico III*, *Ventura* and *Mechest* varieties and *Kapiya* peppers.

Groups with various stages of maturity, comprising 20 fruits each, were formed. The spectral transmittance characteristics of the whole fruits were measured with the spectrophotometer SFP 1 (KRIVOSHIEV *et al.*, 1977) in the visible range, and of some groups also in the infrared range. After this, five independent experts classified the fruits as to their stage of maturity and suitability for a certain processing.

In order to find an index for quick quality evaluation of peaches in the production of peeled peaches, after the measurement with the SFP 1, the peaches were peeled by dipping into a 5% NaOH solution at 368 K (95 °C) for 1 min. After this, the peaches were ranked by experts as to their flesh colour. The apricots were evaluated by the skin colour, after this the fruits were halved and evaluated by the fruit flesh colour, because both the external and internal fruit colour influence the quality of the canned product.

The colour and ripeness of pears are of major importance for fresh consumption. For this reason, light transmittance data of pears were compared with those of their sensory evaluation (STRANDZHEV *et al.*, 1976). *Bartlett* pears were investigated immediately after picking, while *Bosc* and *Passe Crassane* after an appropriate storage period.

The quality of plums depends largely on their external and internal colour, therefore they were classified by these indices.

The same was the case with strawberries, which, however, were evaluated only by the external colour.

After the measurement of spectral characteristics, the tomato varieties for fresh consumption (*Extase*, *Panase* C_3F, *Virase*) were classified by their external colour, then they were cut and classified by their internal colour. The internal state is connected with the ability of tomatoes to ripen after picking in favourable conditions (CHALUKOVA *et* al., 1978; WORTHINGTON, 1974), therefore information on the internal fruit colour is needed as well.

The tomato varieties for processing were investigated by another method, since the colour of the obtained juice is the most important quality index Ten groups of four fruits each were chosen. The light transmittance of each fruit was measured and each group was processed into juice. The colour of the raw juice was measured with a *Gardner* C4 colorimeter. Transmittance data of the whole fruits were correlated with the values of the a_L/b_L ratio of the juice.

The light transmittance data of *Kapiya* peppers were correlated with the visual evaluation of the external pepper colour which determines the quality of fresh and frozen peppers as well as some other products, *e.g.* pickled *Kapiya* peppers.

From the spectral transmittance characteristics obtained, $T_{\lambda i}/T_{\lambda j}$ ratios were calculated, where $T_{\lambda i}$ and $T_{\lambda j}$ are the transmittances of a whole fruit at the wavelengths λ_i and λ_j; in the investigated range λ_i and λ_j have definite values. The logarithm of the transmittance ratio at two wavelengths (*i.e.* the optical density difference for two wavelengths) of the whole fruits was correlated with the data for colour, ripeness and technological suitability, obtained as described above.

2. Results and discussion

The Figures 1 to 22 present the spectral transmittance characteristics of whole fruits. Several typical characteristics of fruits at different stages of maturity, as well as of different quality and suitability for processing or fresh consumption are included. The considerable transmittance differences in some wavelength ranges allow the use of transmittance for non-destructive fruit evaluation.

The spectral behaviour of the various kinds and sometimes the various varieties has specific features, since each of them is characterized by a respective quantity and proportion of pigments in the skin and the flesh. Regardless of this fact, in some parts of the investigated range of wavelengths, the transmittance change during maturation has a similar character for the various species and varieties, *e.g.* with all fruits (except for frozen strawberries). Transmittance grows during maturation in the range of maximum chlorophyll absorption. The influence of the internal colour on the spectral transmittance is revealed most clearly with *Bosc* pears whose surface is brown regardless of

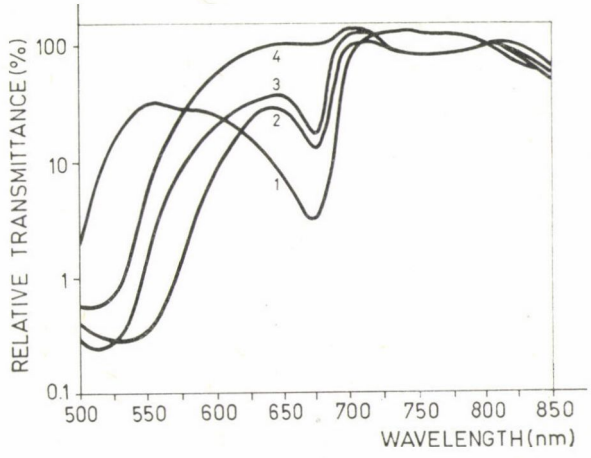

Fig. 1. Spectral transmittance characteristics of *Fortuna* peaches
1–green, 2–unripe, 3–medium ripe, 4–ripe

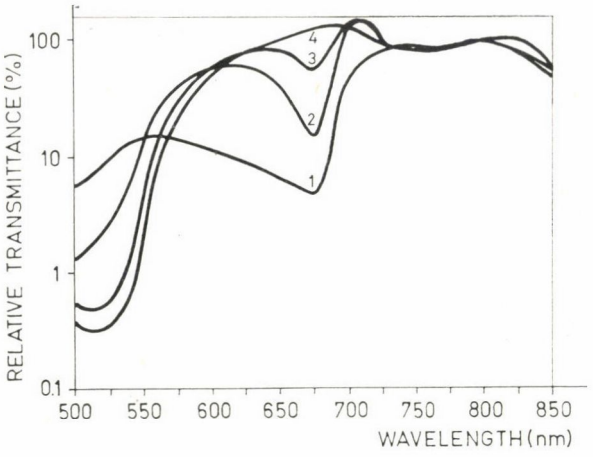

Fig. 2. Spectral transmittance characteristics of *Baby Gold 5* peaches
1–green, 2–unripe, 3–medium ripe, 4–ripe

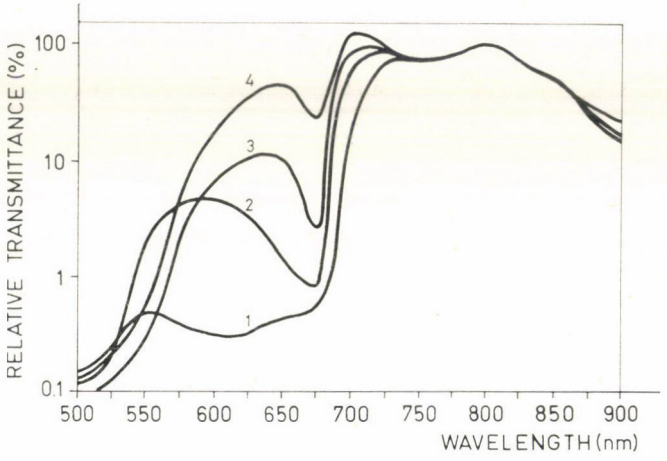

Fig. 3. Spectral transmittance characteristics of *Elberta* peaches
1–green, 2–unripe, 3–medium ripe, 4–ripe

their ripeness. Table 1 presents the rank correlation coefficients (according to *Spearman*) between the transmittance correlations and the data on fruit colour, ripeness and technological suitability. As it is known, the rank correlation methods are preferred in the cases where the classification of objects is carried out by some qualitative index (in this case – stage of maturity) for which no absolute measuring scale exists.

Coefficients of linear correlation were calculated for processing tomatoes, since the quality of the obtained juice was expressed by the values of the

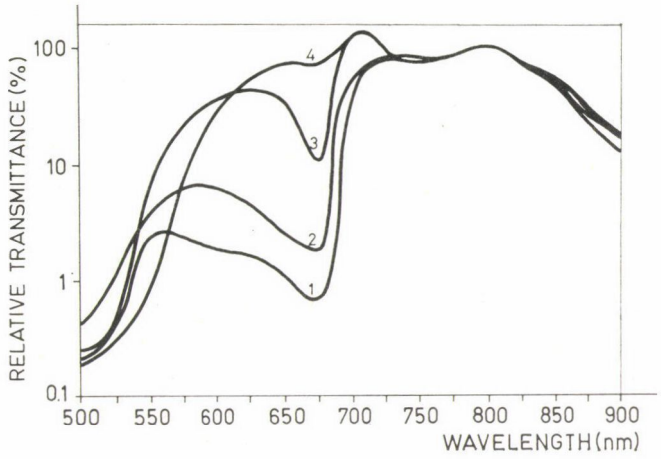

Fig. 4. Spectral transmittance characteristics of *Halle* peaches
1–green, 2–unripe, 3–medium ripe, 4–ripe

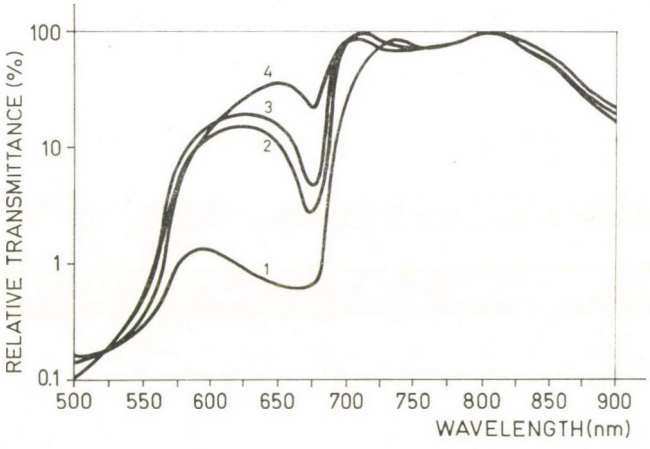

Fig. 5. Spectral transmittance characteristics of *Corona* peaches
1–green, 2–unripe, 3–medium ripe, 4–ripe

colour co-ordinates measured with a tri-stimulus colorimeter. Linear correlation was sought also with apricots, for whose evaluation a colour hue scale was used (KRIVOSHIEV, 1976). Table 1 shows also the values of $\eta_{max} = \frac{(T_{\lambda i}/T_{\lambda j})_{max}}{(T_{\lambda i}/T_{\lambda j})_{min}}$. The higher value of η_{max} allows to distinguish better between fruits of a similar stage of maturation in their evaluation and sorting.

It is evident from the data in Table 1 that the highest correlation coefficient (0.941 to 0.991) and the highest η_{max} values (165 to 219 · 10^3) were found

Table 1

Values of the correlation coefficients between log of the transmittance ratio of whole fruits for the wavelengths λ_i and λ_j and the stage of maturity

Species and variety	λ_i (nm)	λ_j (nm)	Coefficient of rank (ϱ) or linear (r) correlation	$\eta \max = \frac{(T_{\lambda i}/T_{\lambda j})\max}{(T_{\lambda i}/T_{\lambda j})\min}$
1	2	3	4	5
Peaches				
Fortuna	750; 775	650	$\varrho = -0.860$	52
Baby Gold 5	750; 800	675	$\varrho = -0.914$	24
Elberta	750; 775	650	$\varrho = -0.887$	100
Halle	750; 775	675	$\varrho = -0.940$	98
Corona	750; 775	650	$\varrho = -0.896$	49
Apricots				
Magyar Boys	650	550	$r^*_{ext} = 0.920$ $r^{**}_{int} = 0.920$	335
Pears				
Bartlett	650	725	$\varrho = 0.959$	91
	675	750	$\varrho = 0.902$	125
Bosc	650	725	$\varrho = 0.812$	5.4
	675	750	$\varrho = 0.889$	28.7
Passe Crassane	650	750	$\varrho = 0.818$	20.4
	675	750	$\varrho = 0.838$	90.5
Plums	725 to 800	575	$\varrho = 0.988$	43.3 to 47.4
	725 to 800	600	$\varrho = 0.988$	8.3 to 9.2
Greengages	700 to 775	600	$\varrho = -0.990$	5.3 to 15
	700 to 775	625	$\varrho = -0.990$	8.7 to 24.8
Strawberries				
Gorella	700 to 800	575	$\varrho = 0.931$	103 to 114
(frozen)	700 to 800	600	$\varrho = 0.895$ to 0.926	20 to 21.7
Tomatoes				
Extase	625 to 675	550	$\varrho^*_{ext} = 0.941$ to 0.955 $\varrho^{**}_{int} = 0.943$ to 0.953	280 to 1365
	650; 675	575	$\varrho_{ext} = 0.938$; 0.950 $\varrho_{int} = 0.938$; 0.946	165; 388
Panase C_3F	625 to 675	550	$\varrho_{ext} = 0.947$ $\varrho_{int} = 0.965$ to 0.946	181 to 6450
	650 to 675	575	$\varrho_{ext} = 0.975$ $\varrho_{int} = 0.965$; 0.953	1528; 1835
Virase	625 to 675	550	$\varrho_{ext} = 0.985$ to 0.988 $\varrho_{int} = 0.985$	888 to 1418
	650; 675	575	$\varrho_{ext} = 0.990$; 0.986 $\varrho_{int} = 0.984$; 0.979	676; 1280
VF 316	675	575	$r = 0.992$	$52 \cdot 10^3$
VF 198	675	575	$r = 0.979$	$39 \cdot 10^3$
VF 145	675	575	$r = 0.959$	$219 \cdot 10^3$
Chico III	675	575	$r = 0.970$	$4 \cdot 10^3$
Ventura	675	575	$r = 0.989$	$18 \cdot 10^3$
Mechest	675	575	$r = 0.991$	$193 \cdot 10^3$
Peppers				
Kapiya	675	550	$\varrho = 0.979$	$4.8 \cdot 10^3$
	675	575	$\varrho = 0.962$	$3 \cdot 10^3$

r^*_{ext}, ϱ^*_{ext} = coefficient of linear resp. rank correlation with the external fruit colour (skin colour)

r^{**}_{int}, ϱ^{**}_{int} = coefficient of linear resp. rank correlation with the internal fruit colour (flesh colour after cutting)

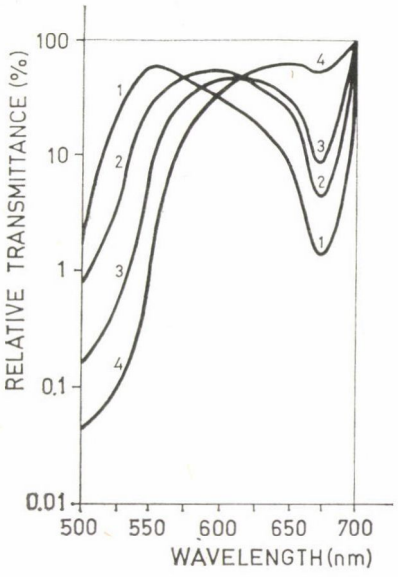

Fig. 6. Spectral transmittance characteristics of *Magyar Boys* apricots
1–yellow-green, 2–yellow, 3–orange, 4–orange-red

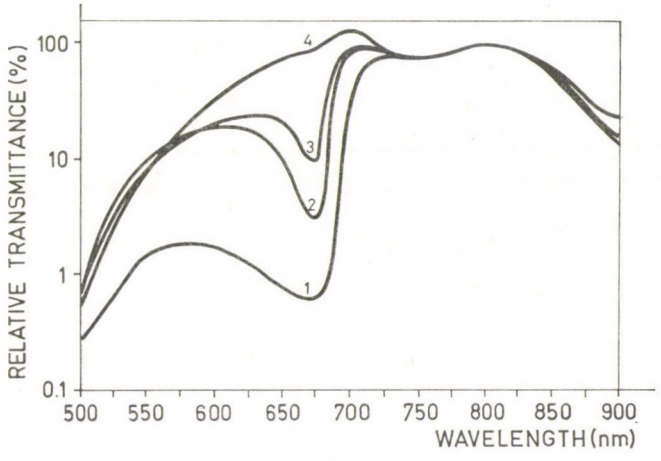

Fig. 7. Spectral transmittance characteristics of *Bartlett* pears
1–green, 2–medium ripe, 3–ripe, 4–over-ripe

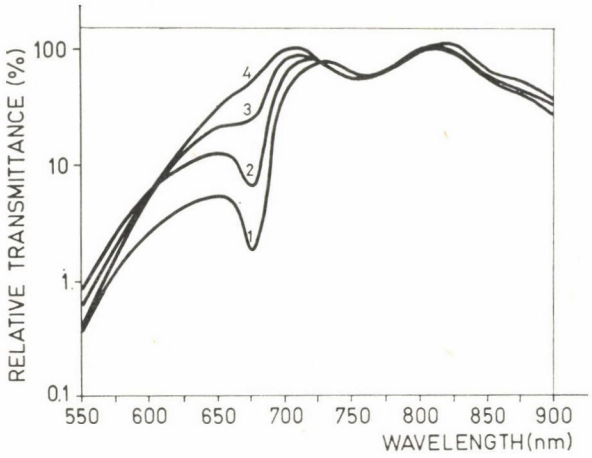

Fig. 8. Spectral transmittance characteristics of *Bosc* pears
1–green, 2–medium ripe, 3–ripe, 4–over-ripe

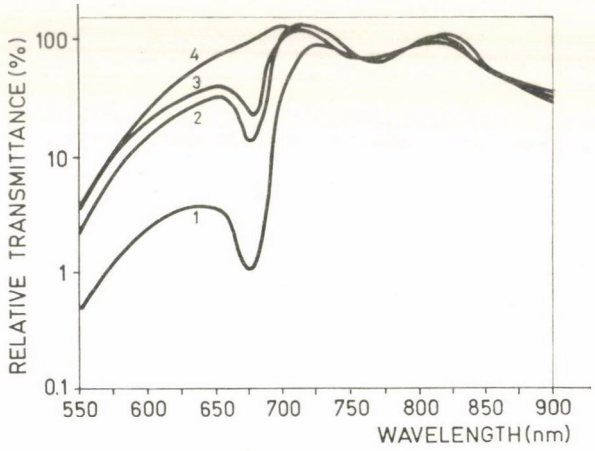

Fig. 9. Spectral transmittance characteristics of *Passe Crassane* pears
1–green, 2–medium ripe, 3–ripe, 4–over-ripe

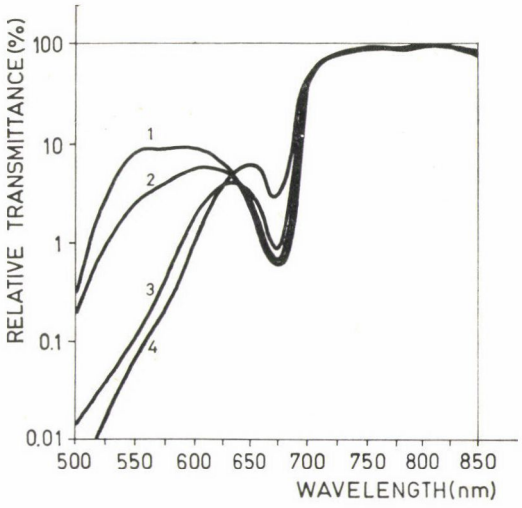

Fig. 10. Spectral transmittance characteristics of plums
1–green, 2–unripe, 3–ripe, 4–over-ripe

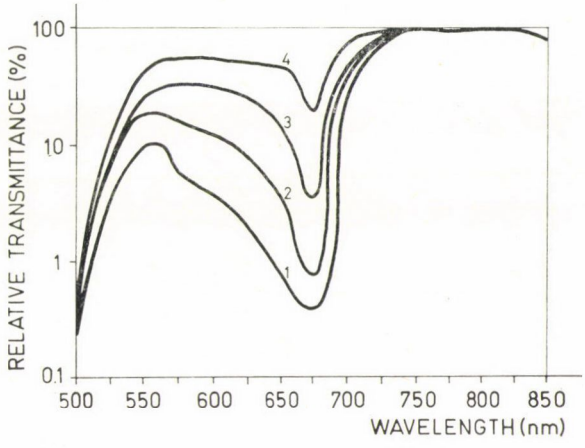

Fig. 11. Spectral transmittance characteristics of greengages
1–green, 2–unripe, 3–ripe, 4–over-ripe

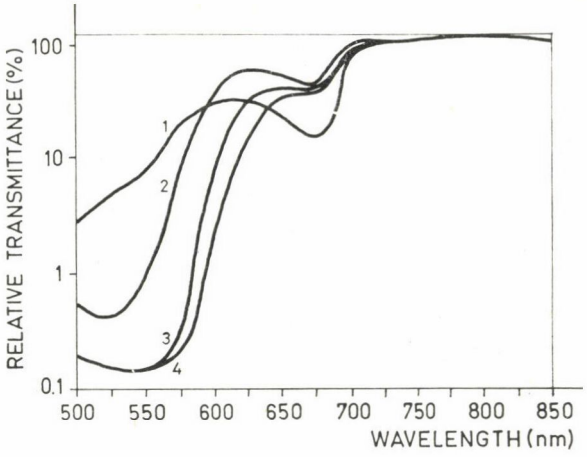

Fig. 12. Spectral transmittance characteristics of *Gorella* strawberries (frozen)
1–yellow-greenish, 2–pink, 3–red, 4–dark-red

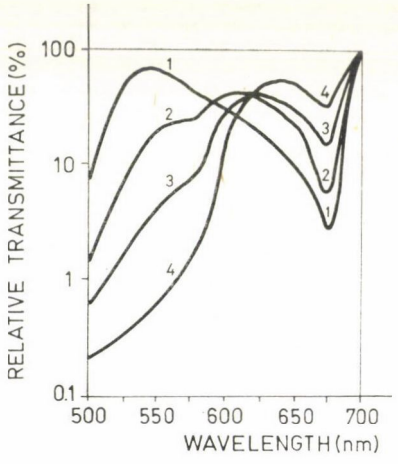

Fig. 13. Spectral transmittance characteristics of *Extase* tomatoes
1–yellow-green, 2–yellow-orange, 3–orange, 4–orange-red

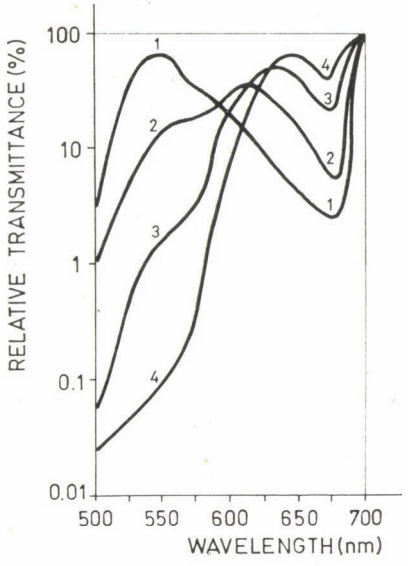

Fig. 14. Spectral transmittance characteristics of *Panase* C_3F tomatoes
1–yellow-green, 2–yellow-orange, 3–orange, 4–orange-red

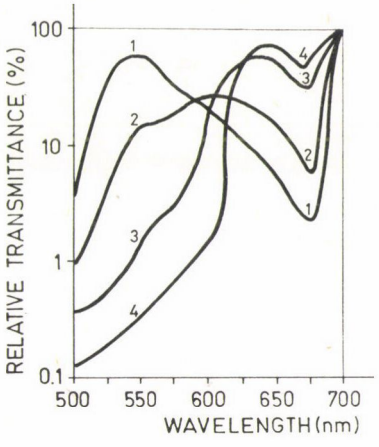

Fig. 15. Spectral transmittance characteristics of *Virase* tomatoes
1–yellow-green, 2–yellow-orange, 3–orange, 4-orange-red

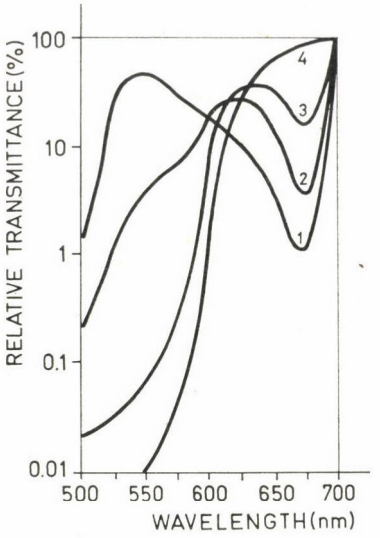

Fig. 16. Spectral transmittance characteristics of VF 316 tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

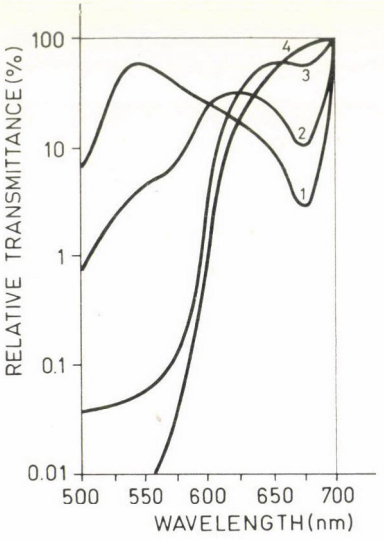

Fig. 17. Spectral transmittance characteristics of VF 198 tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

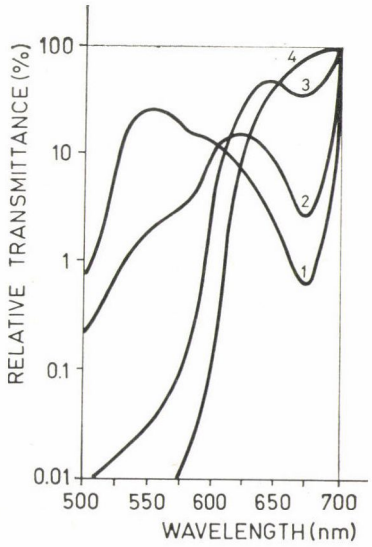

Fig. 18. Spectral transmittance characteristics of VF 145 tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

Fig. 19. Spectral transmittance characteristics of *Chico* III tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

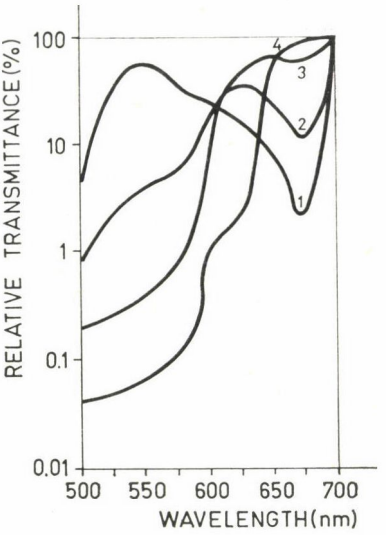

Fig. 20. Spectral transmittance characteristics of *Ventura* tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

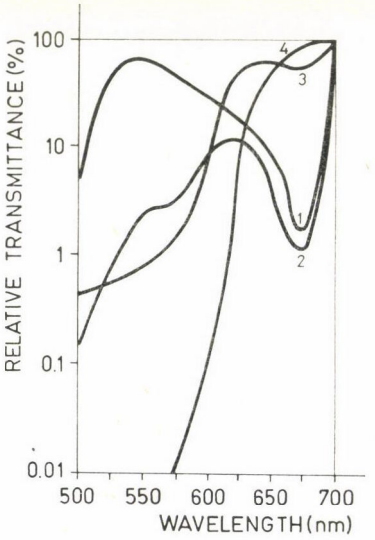

Fig. 21. Spectral transmittance characteristics of *Mechest* tomatoes
1–yellow-green, 2–yellow-orange, 3–orange-red, 4–dark-red

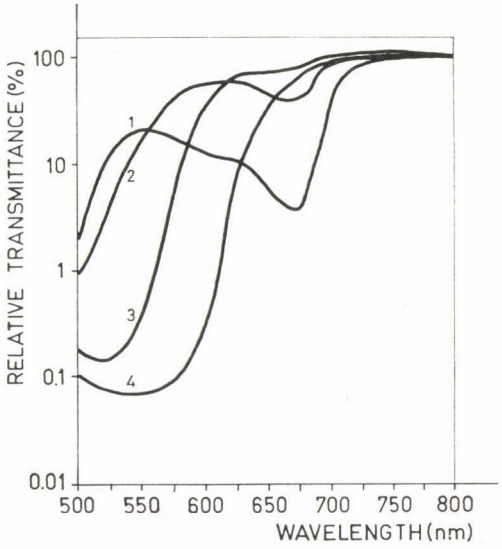

Fig. 22. Spectral transmittance characteristics of *Kapiya* peppers
1–green, 2–yellow-orange, 3–orange-red, 4–dark-red

with tomatoes whose colour hue changed from yellow-green into dark-red. Lower correlation coefficients were found with peaches (–0.887 to –0.940) whose colour hue changes from yellow-green into yellow-orange (η_{max} from 2.5 to 100). The uneven colouring of peaches and apricots also led to a lower correlation.

With pears (except for the *Bartlett* variety) the correlation coefficients are considerably lower (0.812 to 0.889), which to some extent is due to the fact that the fruit flesh colour varies in a small range during ripening. These values however, as well as the η_{max} values (5.4 to 90.5) allow to apply the biochromatic method also with these varieties. The wavelengths given in Table 1 were used in automatic sorting with the Bulgarian colour sorting unit ASC 1/11. The glass-house and field tomatoes for fresh consumption were sorted into four colour fractions, and the mechanically harvested tomatoes for processing, as well as apricots and peaches into three fractions. The sorting error did not exceed 5%.

3. Conclusions

Information on the colour and ripeness of the investigated fruit is found in the range of 525–750 nm.

Chlorophyll reduction during fruit ripening is expressed by a transmittance increase at 675 nm. For this reason, it is suitable for the biochro-

matic evaluation of peaches, pears, tomatoes and peppers by the degree of their maturity.

For evaluating and sorting fruit of the same species but different variety a pair of wavelengths may be chosen, *e.g.*: for peaches 650 (or 675) and 750 nm; apricots 650 and 550 nm; pears 675 and 750 nm; plums and frozen strawberries 600 and 725 nm; tomatoes and peppers 675 and 575 nm.

The correlation coefficients and η_{max} are high enough, therefore the transmittance control at the chosen wavelengths can be used for the quick ripeness evaluation of raw materials and for the development of sorting machines.

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MECHANICAL CHARACTERISTICS OF APPLE TISSUE IN JUICE PRODUCTION

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In the case of low mechanical stress the apple tissue suffers reversible deformation following *Hooke*'s law. The elasticity modulus under uni-axial pressure or bending under a given force was determined in fresh, and stored apple and apple heated to various temperatures. The apple varieties used were: *Jonathan, Golden Delicious* and occasionally *Starking*. Conclusions were drawn on the elasticity modulus alsoby measuring the diameter of the apple slice of a given size not yet braking. Since the latter depends also on tensile strength, this, too was measured and accounted for. Results are given in Table 1 and Figs. 6 and 7. The elasticity modulus of apple flesh was found to vary within a wide range. Comparison with data from the literature reflects differences caused by variety, storage conditions and state of turgor. Substantial effects of heat, even at low heat loads, may be traced back to changes in turgor (Fig. 6). At higher temperature, break down of the tissue started. The systematic measurement of the elasticity modulus facilitates the establishment of the technologically most advantageous mode of heating in apples of different past record. Assays into this subject will be described later.

The main mass of the edible part (flesh) of apple consists of parenchymal storage cells. The mechanical characteristics, texture is determined by the following factors (Lásztity *et al.*, 1973):

– The relative amount and solidity of the cell walls. The amount decreases with growing size; solidity is determined by the quantity of cellulose and protopectin and the state of the latter (Tavakoli & Wiley, 1968).

– The state of the cells and their contents, primarily turgor and the quality and quantity of the starch granules.

– The relative volume and gas content of the intercellular ducts.

In the case of low mechanical stress, the apple tissue, similarly to solid construction materials, suffers mainly reversible deformation, the extent of which is independent of the deformation rate and depends entirely on the measure of stress. The deformations follow *Hooke*'s law. Thus, the elastic behaviour of apple may be characterized by the elasticity modulus (Mohsenin & Göhlich, 1962; Somers, 1965; Tóth, 1971; Nikolaev *et al.*, 1973; Peleg & Calzada, 1976; Chetvertyakov *et al.*, 1977; Van Lancker *et al.*, 1977; Nikolaev & Troyanskaya, 1978).

Upon more extensive mechanical forces, the stress reaches the bio-yield point and the changes in the tissue become irreversible both within and between cells. With increasing force, the advance of deformation is slowing down and

its extent depends on the rate of deformation. The flesh of apple behaves like a viscoelastic body.

When the stress discontinues, the bio-yield point may be characterized by relaxation (SOMERS, 1965; PELEG & CALZADA, 1976; VAN LANCKER *et al.*, 1977).

Further increase of mechanical load may lead to stresses under which the continuity of the tissue is broken at the macro level. This stress is considered the solidity of the fruit flesh. The extent of this stress depends largely on the rate of load or deformation.

The mechanical characteristics of apple are tested from different practical points of view (VAN LANCKER *et al.*, 1977), such as:

– Determination of the optimum harvest time. To determine this either the whole apple with skin is exposed to mechanical force or the skin is removed from the surface tested (HÁMORI-SZABÓ, 1974) or test pieces removed from the flesh are investigated (NIKOLAEV & TROYANSKAYA, 1978). Recently, dynamic methods are suggested to evaluate the viscoelastic characteristics of tissues (MORROW & MOHSENIN, 1968).

– Estimation of damage occurring during harvesting, transfer and storage and its limitation. This is tested by static and dynamic stress of the whole fruit (MOHSENIN & GÖHLICH, 1962; HOLT & SCHOORL, 1977; SALASHINSKII, 1973).

– To establish the parameters for the technological operations (TÓTH, 1971). In this study the apple was tested from this point of view.

Generally, for solvent–water extraction, the apple slices are made permeable by temperature increase. This accelerates at the same time the mass transfer in the fruit out into thin slices (OTT *et al.*, 1960; KARDOS, 1974; LÜTHI & GLUNK, 1974; VUKOV & MONSZPART-SÉNYI, 1977).

If the apple juice is to be gained by pressing, the fruit is comminuted and the pulp is heated and/or treated with pectolytic enzyme. The juice is pressed out of the intercellular spaces (KÖRMENDY, 1965; KARDOS, 1979). The juice yield of a given raw material depends on the way of preparation, however, some of the parameters are hardly dependent on the preparation but are characteristic of the mechanical properties of the pulp (MOLNÁR, 1971; 1973).

In view of extraction by diffusion, the deformability of the raw and the heated apple tissue is important. This is characterized by the elasticity modulus and the bending radius of the slices. The aim of this study was to determine the elasticity modulus and the critical bending radius of raw and heated apple tissue, in order to establish the deformation as caused by the diffusion technique of juice extraction.

1. Materials and methods

1.1. Materials

The apples used in the experiments belonged to well defined varieties and were stored separately in commercial storehouses. In November 1976 and in March 1977, *Jonathan* and *Golden Delicious* and in the autumn of 1977 and 1978 the variety *Starking* were studied. During 1979, apples of the above varieties, picked at three different times from the same trees were used.

The test pieces of various sizes and shapes (cylinder, planparallel slices) were cut out of the flesh of the apples and were warmed in distilled water preheated in a thermostat. The proportion of water to the test piece was between 2.5 : 1 and 10 : 1, in order to warm them as rapidly as possible.

1.2. Methods

1.2.1. Determination of the elasticity modulus. The classical method to measure the elasticity modulus in plant tissue is the pressure test. This may be carried out in two different ways with test pieces of different sizes. The simpler of the two is the measurement of deformation as caused by the load. However, the method based on deformation upon the given rate and simultaneous measurement of the force is suitable to determine not only the elasticity modulus but also the deformations and flow (MOHSENIN, 1970; BIRÓ *et al.*, 1977; VAN LANCKER *et al.*, 1977). The measurements of the test pieces described in the literature are: cylindrical bodies of diameter 38 mm, height 12.7 mm (MOHSENIN & GÖHLICH, 1962); diameter 11 mm, height 11 m (NIKOLAEV *et al.*, 1973); diameter 12.6 mm, height 10 mm (PELEG & CALZADA, 1976); diameter 15.5 mm, height 25 mm (VAN LANCKER *et al.*, 1977); and a cube of 5 mm edge (SOMERS, 1965). There are several authors, who measure the force belonging to a given deformation with cylindrical or ball penetrometers and calculate the elasticity modulus by the *Poisson* factor (VAN LANCKER *et al.*, 1977; CHETVERTYAKOV *et al.*, 1977).

The other method of determining the elasticity modulus is the flexibility test on cylinders of 8 mm diameter and 50 mm length by loading them as consols of 40 mm (VUKOV, 1956; VUKOV & MONSZPART-SÉNYI, 1977; TÓTH, 1971). This has been the method used in the following experiments. The principle of the method is shown in Fig. 1.

The majority of the results processed here were established by pressure test measuring the deformations formed under a given load. A *Höppler* consistometer with a plastometer fitting (HÖPPLER, 1940) was used for this purpose. The test piece of 1.13 cm diameter and 1 cm height is placed between two rustproof metal cylinders, conducted nearly attrition-free. Compression formed upon discretely variable forces, is measured. The basic load is 2.45 N. The following loads were applied: 4.90 and 9.81 N. Since the cross-section of

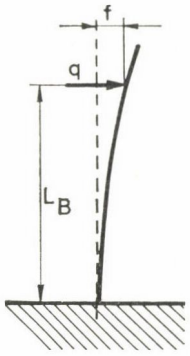

Fig. 1. Determination of the elasticity modulus by the flexibility test. Useful length of the test piece L_B (40 mm), q flexing force, f deformation

the test piece is 1.0 cm^2, the force was obtained in N cm^{-2} units. Deformation of the test pieces was almost reversible. The lasting deformation may be explained by the ruggedness of the surface. The deformation may be followed on the instrument to 0.01 mm accuracy. Results were read 1 min after loading or unloading. Changes in the height of the test piece with time are shown on an example in Fig. 2.

Starting from the instant of loading, the final extent of compression was reached always within one min. Upon unloading, deformation is mostly reversed, dependent on the elasticity modulus. The elasticity modulus was calculated from the diminishing of deformation upon unloading, thus, the non-reproducible lasting deformation of unknown origin was omitted.

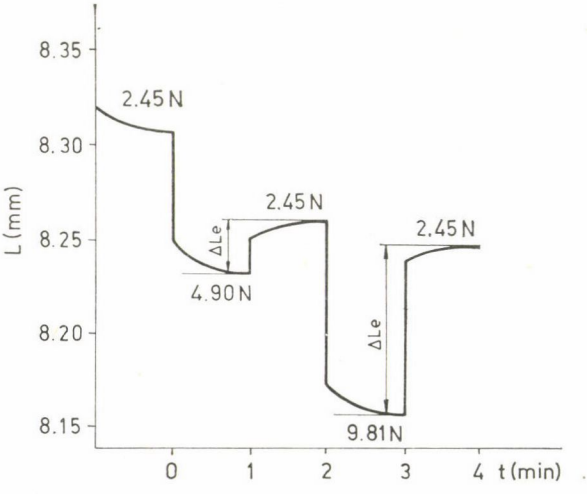

Fig. 2. Pressure test: change in the height of the test piece (h) under pressure and after release as a function of time (t)

At higher loads, the deformation grows with time and the material breaks. According to the measurements of the authors, if apples are correctly stored, this does not occur even at a stress of 0.01 MPa. However, at a stress of 0.025 MPa it occurs at all events.

1.2.2. Determination of flexibility. The flexibility of apple slices may be measured by the *Irion* test (DRATH, 1976). Appropriately thin slices (of a thickeness of a mm are cut and are exposed to bending between solid cylinders of diameter D mm. In the course of bending, the planparallel slices are moved from the larger to the smaller diameter till they brake. The diameter where this occurs is the critical diameter: D_{cr}.

According to the original concept, the flexibility test was carried out between a series of cylinders of diminishing diameter, manually. Based on the design of András ZSIGMOND an appliance was constructed the main part of which is a horizontal truncated cone, having all the necessary diameters and the slice is bent by placing a superficies, corresponding to the negative of the cone, over it. Thus, several test pieces may be flexed at the same time and the critical diameter needs little time to be established. The main dimensions of the appliance are shown in Fig. 3.

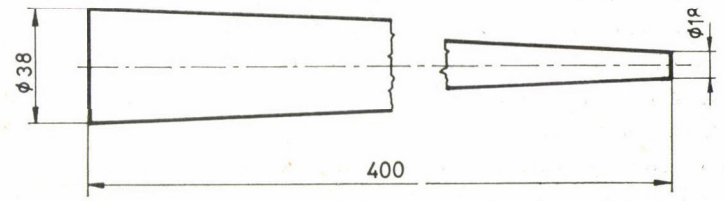

Fig. 3. Main dimensions of the cone used in the flexibility test (in mm)

The principle of the flexibility test is illustrated in Fig. 4.

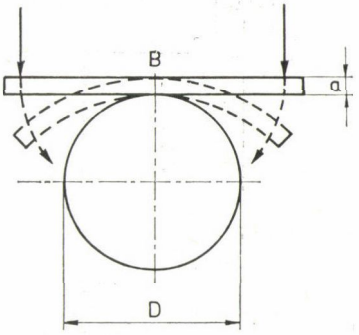

Fig. 4. Principle of the flexibility test: D flexing diameter, a thickness of the test piece

The maximal stress (σ_B) in point B is formed in the outside zone. This is expressed by the following formula (based on relations known from the science of elasticity)

$$\sigma_B = \frac{12a}{\pi^2} \cdot \frac{E}{D}$$

where: a is the thickness of the apple slice (test piece) and E the elasticity modulus. As may be seen, the stress in a body of given elasticity is in inverted proportion to diameter D. In the case of diameter D_{cr}, the slice cracks or breaks. Of the measures in the equation D and a may be measured by length. Given tensile strength σ_1, elasticity modulus E may be computed and *vice versa*.

In these experiments, the thickness of the slice was $a = 2.0$ mm; its width 55 mm. Thus

$$\sigma_B = 2.43 \cdot 10^{-3} \frac{E}{D}.$$

(If the force is expressed in MN, σ_B is obtained in MN m^{-2} units.) Given the tensile strength σ_1 (determined by tensile test) and elasticity modulus E (determined by the flexibility test), between the σ_B value calculated by the above equation and the critical diameter (D_{cr}) a relatively good correlation was found, $r = 0.71$. A better correlation could not be expected for theoretical reasons, since the unevenness of the surface of the flexed slice affects seriously the critical diameter.

The flexibility test, as described above, is an adequate means to determine the suitability of the apple to extraction by diffusion.

Since flexibility is strongly dependent on tensile strength, the latter was established by tensile test on test pieces of biscuit-finger shape and the tensile force was determined. The shape and size of the test piece are shown in Fig. 5.

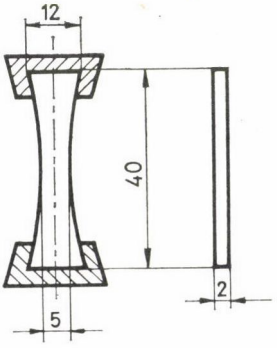

Fig. 5. Test piece used in the tensile strength test (in mm)

2. Results

2.1. Elasticity modulus

The values as determined by pressure and flexibility test in raw apple flesh differ. Those measured by flexibility test are generally lower. It is assumed that, in the course of flexibility testing, the test pieces loose moisture and this is accompanied by the reduction of turgor and, in consequence, a reduction in the value of the elasticity modulus. Average values are given in Table 1.

Table 1

Elasticity modulus (E) of raw apple, stored for a short time, measured by different methods

Method of determination	*E* (MPa)			
	Average	Standard deviation	Extreme values	
Pressure test	1.22	±0.32	0.4	2.2
Flexibility test	0.6		0.3	1.2
Flexibility test	0.68	±0.18	0.45	0.98

In 1979 the elasticity moduli were separated according to apple variety. The mean values, their deviations and their level of significance are given in Table 2.

Table 2

Elasticity modulus (E) of different apple varieties

Time of picking: September 25, 1979

Variety	*E* (MPa)		Level of significance
	Average	Standard deviation	
Jonathan	0.73	±0.15	not significant
Starking	0.43	±0.03	$P < 0.05$
Golden Delicious	0.68	±0.10	

Similar data, related to apples picked at different times are contained in Table 3.

During warming in water the change in the elasticity modulus is due first to the increased turgor pressure, the consequence of water absorption,

Table 3

Elasticity modulus (E) as affected by the time of picking

Date of picking	E (MPa)		Level of significance
	Average	Standard devialion	
1979—09—05	0.68	±0.07	
1979—09—15	0.43	±0.04	$P < 0.05$
1979—09—25	0.71		$P < 0.05$

then to the change of permeability in the membranes. The slight increase and the subsequent extensive decrease of the elasticity modulus at various temperatures *vs.* time is illustrated in Fig. 6.

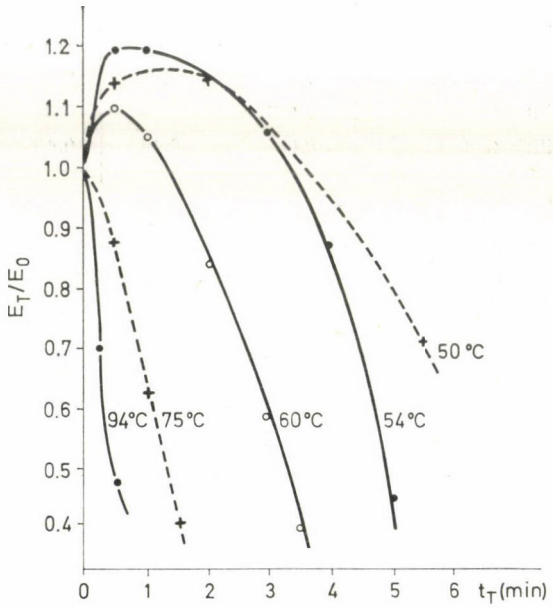

Fig. 6. Relative values (E_T/E_0) of the elasticity modulus of apple flesh after treatment at various temperatures (T) as a function of the heating period (t)

It may be seen in the Figure that at temperatures 50, 54 and 60 °C the elasticity modulus increases in the first minutes with the swelling of the tissue, then it decreases at a rate depending on the temperature. At higher temperatures the decrease is monotonous and at 94 °C it tends to very small values.

2.2. Flexibility

Flexibility measurements were carried out with *Jonathan* apples, only, freshly picked (I) and after 63 days storage at 10 °C (II). The frequency of the critical flexibility diameters D_{cr} and tensile strength values belonging to them, as established from 45 measurements, are shown in Fig. 7.

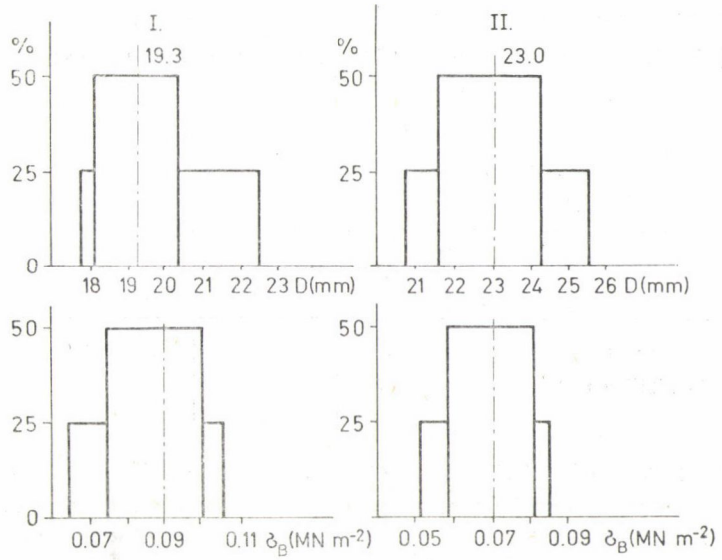

Fig. 7. Frequency of the critical diameters (D, mm) and of tensile strength (σ_B): (I) Jonathan, fresh (II) Jonathan, stored at 10 °C for 63 days

As it may be seen in the histogram, the most frequent diameter with fresh apples is 19.3 mm, while in stored apple 23.0 mm. The tensile strength in fresh apples amounts to 0.09 MPa, while in stored apple to 0.07 MPa.

The average values of the elasticity modulus as calculated from two series of measurements: for fresh apples (I)

$$E = 0.72 \pm 0.12 \text{ MPa}$$

for stored apples (II)

$$E = 0.65 \pm 0.13 \text{ MPa}$$

The average of the two series: $E = 0.68 \pm 0.18$ MPa. This value is given in Table 1, too. After the sign $\pm$ the deviation belonging to one result is given. The deviation of average values (I) and (II) and the error of the deviation: $\Delta E = 0.07 \pm 0.02$ MPa; significant at level P = 0.01. In the course of heating the flexibility of the apple substantially increases, thus, the critical diameter is always below 10 mm.

3. Conclusions

In evaluating the results obtained by measuring the elasticity modulus of raw apple, data found in the literature can be used for comparison. These data were obtained by different methods and expressed in different units. Only data obtained by exact measurement of the elasticity modulus were taken into account and these were converted to MPa units.

Flexibility data were published only by TÓTH (1971). MOHSENIN & GÖHLICH (1962), NIKOLAEV *et al.* (1973), NIKOLAEV & TROYANSKAYA (1978),

Table 4

Mean values of the elasticity moduli in apple flesh on the basis of data of different authors

Method of determination	E (MPa)			
	Average	Standard deviation	Extreme values	
Pressure test	3.1	± 1.8	0.7	6.9
Flexibility test	0.7			

CHETVERTYAKOV *et al.* (1977) and VAN LANCKER *et al.* (1977) obtained their results in pressure tests. Average values, deviations and extreme values belonging to data taken from the above sources are contained in Table 4.

Data given in Table 1 fall between the limits of deviations as shown in Table 4, they are, however, lower than the averages found in the literature. It may also be seen in Table 4 that elasticity moduli vary between wide boundaries. Of the factors affecting scatter, the following were studied:

Differences due to variety: as seen in Table 2 these a.e of the order 0.3 MPa. Data of NIKOLAEV, having higher absolut values, show corresponding effect of variety (for *Jonathan* 4.1 $\pm$ 3.5, for *Ranet Simarenko* 3.7 $\pm$ 0.04 MPa, non-significant). The data calculated from the results given by NIKOLAEV and TROYANSKAYA (1978) showed significant differences between varieties (Table 5) and these corresponded to the above said: the extensive scatter may not be explained by differences in variety.

Differences due to degree of maturity: according to the data given in Table 3, the differences due to different stages of maturity are of similar extent as those due to varieties. The results obtained by NIKOLAEV and TROYANSKAYA (1978) in similar experiments do not show changes of consistent character. The difference between green and yellow *Golden Delicious* apples, as calculated from the results of VAN LANCKER and co-workers (1977) was non-significant. Thus the degree of maturity is not responsible for the extensive differences as observed in the elasticity moduli.

Table 5

Elasticity modulus (E) in two apple varieties
(Based on data by NIKOLAEV & TROYANSKAYA, 1977)

Variety	*E* (MPa)
Antonovka	1.99
Pepin Shafrannyi	2.21
Deviation	0.22—0.06*

* Significant at $P < 0.05$

Differences due to storage: the effect of storage on the elasticity modulus of apple flesh was systematically studied by NIKOLAEV and co-workers (1973). These results were processed and are contained in Table 6. The deviation as seen in this Table are substantially higher than those of about 0.1 MPa as described in section 2.2.

Table 6

Diminishing of the elasticity modulus of apple during storage
(Based on data by NIKOLAEV *et al.*, 1973)

Storage time and relative humidity	*E* (MPa)	
	Stored at	
	+5 °C	+20 °C
Initial	3.90	3.90
stored at $\varphi = 95\%$	2.65	1.70
$\varphi = 84\%$	1.44	0.92

During the storage of apples, enzymic processes, dependent mainly on temperature and composition of the atmosphere, exercise a diminishing effect on the elasticity modulus. From the data of Table 6, it appears that the diminishing of elasticity is mainly due the loss of moisture, followed by the reduction of turgor.

Summing up the evaluation of results related to the elasticity modulus of raw apples it may be said that the most extensive differences are caused by the state of turgor. The differences of the proportion of 1 : 10 may be explained only by this, just as it was found in relation to the storage tissues of sugar beet (VUKOV, 1956, 1958; VUKOV & MONSZPART-SÉNYI, 1977).

Thus, the elasticity modulus is the measure of turgor. The elasticity modulus of turgescent apple is 1.2 MPa or above, while that of non-turgid apple 0.4 MPa or below.

When treated in warm water, up to not too high temperatures, the elasticity modulus of the apple flesh increases while, upon water absorption through the semi-permeable plasma membrane, its tissue becomes inflated. When the structure of the plasma breaks down as caused by high temperature, and the membrane becomes permeable, the state of turgor ceases to exist and the elasticity modulus rapidly diminishes. The same phenomenon was observed earlier in the tissue of sugar beet (VUKOV, 1956, 1958). Similar data related to apple were first published by TÓTH (1971). He found the elasticity modulus of test pieces cut from the flesh of apple to fall below the measuring limit (cca 0.1 MPa). He established also that when the test pieces were wetted with milk of lime and washed in distilled water, their elasticity modulus increased substantially, to 1.8 from 0.7 MPa. In sugar beet, this increase was somewhat lower (from 3.4 to 4.7 MPa) and this effect was mostly retained during heating in water (0.48, 0.55 MPa, resp.).

It may be said, therefore, that the elasticity modulus is characteristic not only of the state of turgor in raw apples, but of its change, as well.

The diminishing of the elasticity modulus on heating explains also the substantial increase in the flexibility of apple slices. This is valid even in the case when the tensile strength of the apple size also decreases upon heating as was observed in measurements not published.

Apart from the well-known fact that the mechanical characteristics of plant tissues, thus of apple, too, vary within a wide range, it may be concluded from the results of these experiments that, given the origin of the raw material, of its storage and of its pretreatment, the expectable mechanical characteristics may be predicted. The effects of technological treatments may be predicted similarly. To achieve this, the described measurements have to be carried out regularly and thus a scientifically founded basis is obtained for the most advantageous technology.

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THE CORRELATION BETWEEN QUALITY PARAMETERS AND OPTICAL TRANSMITTANCE OF SOME STONE FRUITS DETERMINED WITH A NEAR-INFRARED COMPOSITION ANALYSER

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Preliminary studies were made on cherry, sour-cherry, apricot and plum samples to establish how quality parameters can be determined by OD measurements and to estimate the accuracy in predicting the quality parameters of the above stone fruits using the instrumental method mentioned.

A *Neotec* 6450 type research composition analyser was used to study samples received at three different phases of the ripening period. On each occasion, fruits of 4 different degrees of ripeness were examined (5 pieces for each degree of ripeness).

The characteristic pairs of wavelengths for the four fruit species and the standard error of estimate for the predicted quality parameters derived from transmission OD values, as measured at the characteristic wavelengths, compared to quality parameters, were determined.

In the case of sour-cherry, it was possible to predict ripeness values with a standard error of estimate of 2.89% using the OD values obtained from measurements at the wavelengths of 585 and 700 nm. (The standard error of estimate also includes the subjective error for the sensorically determined percentage of ripeness.) The dry matter content could be predicted with a standard error of estimate of 0.44 w% from OD values measured at 585 and 725 nm. The acid content showed a standard error of estimate of 0.08 w%, predicted from OD values measured at wavelengths of 588 and 717 nm. Finally, the sugar content (based on the refractive index) was predicted from OD values measured at 585 and 610 nm with a standard error of estimate of 0.85%.

The above described studies led to the conclusion that, in the case of stone fruits, the quality parameters of major importance can be predicted with a quick, accurate and non-destructive instrumental method using OD values measured at some characteristic wavelengths.

Growing stone fruits in Hungary is of increasing significance especially as a further production increase can be expected deriving from an additional 1000 hectares every year after 1980. Beside a quantitative increase, quality requirements of the market are also growing. To satisfy the different demands of the different users, it is required to predict the quality parameters with a rapid objective method. Producers have long expressed the need for a quick, accurate, non-destructive instrumental method for predicting the quality of stone fruits at the different phases of ripening, for determining the distribution of ripeness on a tree, the effect of chemical treatments, mechanized harvest, transport and storage. The four most important stone fruit species in Hungary are cherry, sour-cherry, apricot and plum. During our prelimi-

nary studies we dealt with these four fruit species in order to elaborate methods and single-purpose instruments for rapid non-destructive quality prediction.

In the United States, research work on instrumental, non-destructive quality prediction of fruits has already been started several years ago at the USDA AGRICULTURAL RESEARCH CENTER, INSTRUMENTATION RESEARCH LABORATORY, Beltsville. Their publications included results on determining internal quality, ripeness, pigment content, internal decay and other properties.

SIDWELL and co-workers (1961) estimated the stage of maturation and chlorophyll content of *Elberta* peaches using light transmittance techniques.

BIRTH and OLSEN (1964) performed OD measurements at different wavelengths to predict the quality of *Delicious* apples.

BITTNER and NORRIS (1968) published studies on the correlation between ripeness and optical properties of fruits.

MCCLURE and co-workers (1975) gave an account of a high-speed sorting device based on OD values measured at different wavelengths.

WATADA and co-workers (1976) suggested using light absorption techniques for determining the chlorophyll and carotenoid contents of fruits and vegetables.

WORTHINGTON and co-workers (1977) as well as WANG and WORTHINGTON (1979) studied ripening of *Eldorado* and *Bartlett* pears measuring transmission and reflection optical properties.

Simultaneously, the RESEARCH INSTITUTE FOR THE CANNING INDUSTRY at PLOVDIV, Bulgaria, also carried out intensive work on determining the quality of fruits and vegetables with a non-destructive instrumental method using OD values measured at different wavelengths. As a result of research work several instruments were developed and applied on high capacity automatic sorting devices. KRIVOSHIEV (1974) and KRIVOSHIEV and co-workers (1977) published the results obtained at Plovdiv.

Most researchers dealing with non-destructive, instrumental quality control suggest using the difference of OD values measured at two "characteristic" wavelengths in order to predict the stage of ripeness.

There are some researchers who reported on two characteristic wavelengths for some fruits without, however, giving the regression equation, the correlation coefficient, or the standard error of estimate characterizing the quality of the measurement. As an exception, we should mention KRIVOSHIEV and CHALUKOVA who have handed over to the present authors their study in which the suggested characteristic wavelengths and correlation coefficients are listed for different fruit species and some varieties within the species.

While some researchers were active in determining the correlation between optical properties and quality parameters of fruits, measuring techniques

in the visible and near-infrared range for measuring transmission and reflection data of materials have been developed at an even greater pace. MASSIE and NORRIS (1971) reported on a high-intensity spectrophotometer interfaced with a computer for food quality measurement. CHEN and NATTUVETTY (1977) have already introduced a portable single-purpose instrument with fiber optics for predicting the stage of ripeness for apples, oranges and tomatoes.

The present study was aimed at giving ready-to-use data for designing a single-purpose instrument for predicting quality parameters of stone fruits with special regard to species grown in the Carpathian Basin. For this purpose the most suitable form of regression equation as well as the characteristic wavelengths had to be found.

1. Materials and methods

Fruit samples were received from the RESEARCH INSTITUTE FOR FRUIT GROWING AND ORNAMENTAL PLANTS, Budapest (henceforth: RIFGOP) in the 1979 seasons. Fruit samples were picked at three different periods of ripening representing four different stages of ripeness, five pieces of each fruit. Thus, 60 pieces of fruit samples from each species (always of the same variety) were examined.

The cherries received were from Dunakeszi (variety *G–1*), sour-cherries from Budaörs (variety *P—141*), apricots also from Budaörs (variety *M–L*) and finally plums from Zsámbék (variety *Beszterce–K*).

The fruit samples were labelled with four quality parameters determined by traditional standard methods at the RIFGOP. These parameters were: percentage of ripeness, dry matter content, acid content and the refractive index. Ripeness was sensorically assessed by a well-trained expert and expressed as percentage of full ripeness; dry matter content was calculated from the weight difference before and after drying at 70 °C to constant weight, and expressed in weight per cent; acid content was determined by titration and calculated as per cent (w/w) citric acid; refractive index was measured with an Abbe refractometer.

The optical properties of fruit samples were measured with the *Neotec* research composition analyser type 6450. Transmission measurements were made by passing the light beam through the whole intact fruit.

The beam of light was parallel to the axis of the fruit sample. The diameter of the diaphragm used for cherries and sour-cherries was 13 mm, for plums 20 mm and for apricots 35 mm, respectively. The spectroanalyzer was working within the 380–760 nm range. The number of OD spectra taken were 20 of each sample (this took 8 seconds), and their averages were stored on a floppy disc for evaluation and further processing.

The OD values obtained at different wavelengths were formed by the spectro-analyser as the logarithm of the ratio of light energy measured on the detector without and with sample.

Data processing was performed with a NOVA III type computer built into the research composition analyser. The correlation between the OD values measured at different wavelengths for each sample and the four quality parameters determined by standard methods at RIFGOP were studied in the form of different mathematical equations. The computer determined the coefficients of the regression equations belonging to OD values measured at different wavelengths, as well as the so predicted quality parameters and their correlation coefficients. Finally, it plotted the correlation spectra as a function of wavelength.

The first characteristic wavelength was chosen at the maximum value of the correlation spectrum. The second characteristic wavelength was determined so that the multiple correlation coefficient reached a maximum. The computer listed the values of quality parameters obtained from RIFGOP and values computed from the regression equations into which the OD values measured at the characteristic wavelengths were substituted as well as their differences. On this basis, the standard error of estimate was determined.

The definition of the standard error of estimate is:

$$\sqrt{\frac{\sum_{i=1}^{n}(Q_{si}-Q_{ci})^2}{n-1-p}}$$

where n is the number of samples, Q_s is the respective quality parameter determined by standard methods, Q_c is the same quality parameter computed from the regression equation, p is the number of terms in the regression equation.

In the rest of this paper, main stress is laid on results obtained in studies performed on sour-cherry.

2. Results

The transmission OD spectra of cherry samples of different ripeness can be seen in Fig. 1, those of sour-cherry samples in Fig. 2, while those of plums in Fig. 6 and those of apricots in Fig. 4. In Fig. 3 and Fig. 5, resp., the differences between the OD spectra of samples of different ripeness referred to the least ripe sample are shown for sour-cherries and apricots, resp.

The Figures clearly show that although the OD spectra of the four fruit species are basically similar, certain characteristic differences can be pointed out. It is evident that the so-called characteristic wavelengths for predicting quality parameters are different for the different fruit species. The shapes of

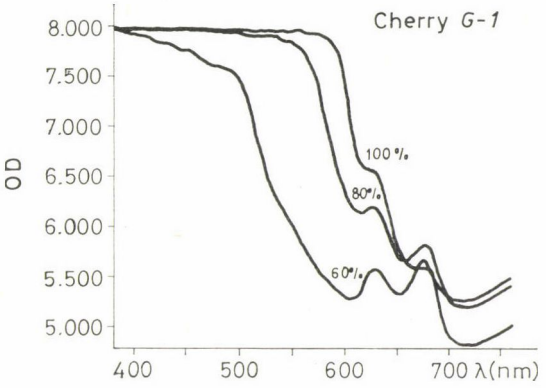

Fig. 1. Transmission OD spectra of cherry samples (variety *G–1* grown in Dunakeszi) for 60, 80 and 100% ripeness. Diameter of diaphragm 13 mm. Illumination parallel to fruit axis

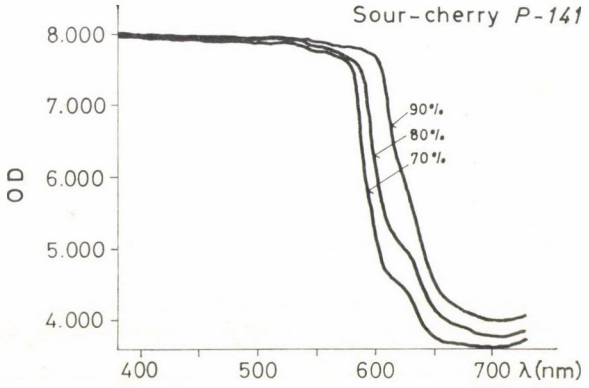

Fig. 2. Transmission OD spectra of sour-cherry samples (variety *P–141* grown in Budaörs) for 70, 80 and 90% ripeness. Diameter of diaphragm 13 mm. Illumination parallel to fruit axis

the spectra show that changes are not only in the direction of the ordinate (OD), but also in the direction of the abscissa (λ); *e.g.* in Fig. 4., the maxima are shifted towards higher wavelengths as ripeness changes.

Repeated measurements with the same fruit sample proved that the reproducibility of OD spectra was the best in case the axis of the fruit was parallel to the beam of light; rotation around the axis hardly influenced the spectra. Defining the reproducibility of OD spectra is, however, very difficult, as it depends on wavelengths, OD values and some other factors. Nevertheless, it can be said that the maximum deviation in the whole wavelength region applied was less then 0.5 OD for a sour-cherry sample placed repeatedly into the instrument for OD spectral analyses.

The research composition analyser sought correlation among the OD spectral values measured at different wavelengths for the 60 samples of each

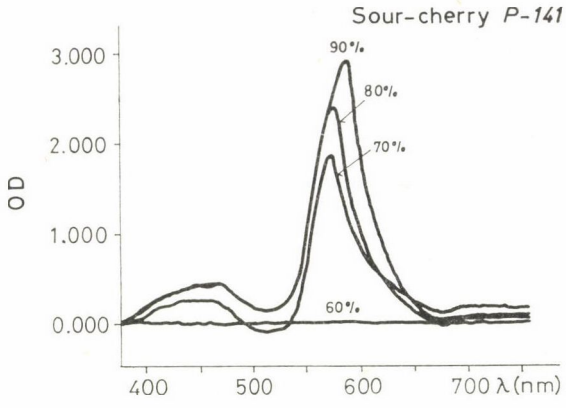

Fig. 3. The relative transmission OD spectra of sour-cherry samples (variety *P–141* grown in Budaörs) of 70, 80 and 90% ripeness related to spectra of sample of 60% ripeness

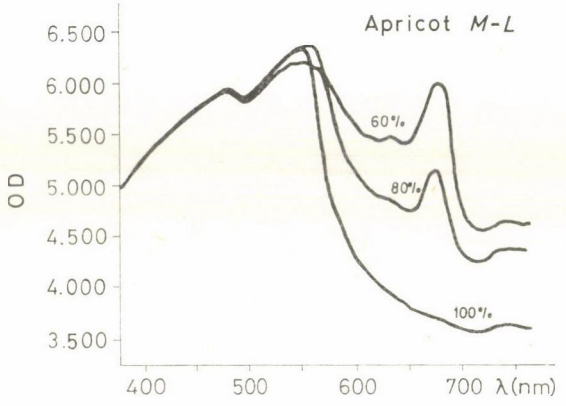

Fig. 4. Transmission OD spectra of apricot samples (variety *M–L* grown in Budaörs) for 60, 80 and 100% ripeness. Diameter of diaphragm 35 mm. Illumination parallel to fruit axis

of the four fruit species as well as the quality parameters received from RIFGOP in the form of the following two equations:

$$Q_1 = k_0 + k_1 \, \mathrm{OD}_{\lambda 1} + k_2 \, \mathrm{OD}_{\lambda 2} \tag{1}$$

$$Q_2 = k_3 + k_4 \frac{OD_{\lambda 3}}{OD_{\lambda 4}} \tag{2}$$

where Q_1, Q_2 stand for quality parameters, $k_0, \ldots, k_4$ are coefficients (constants, resp.), $\lambda_1, \ldots, \lambda_4$ are characteristic wavelengths.

In the search for the first characteristic wavelength referring to ripeness, the instrument determined the correlation between the percentage of ripeness

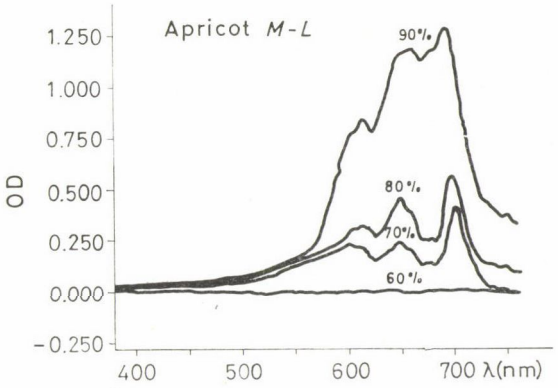

Fig. 5. The relative transmission OD spectra of apricot samples (variety *M–L* grown in Budaörs) of 70, 80 and 90% ripeness related to spectra of sample of 60% ripeness

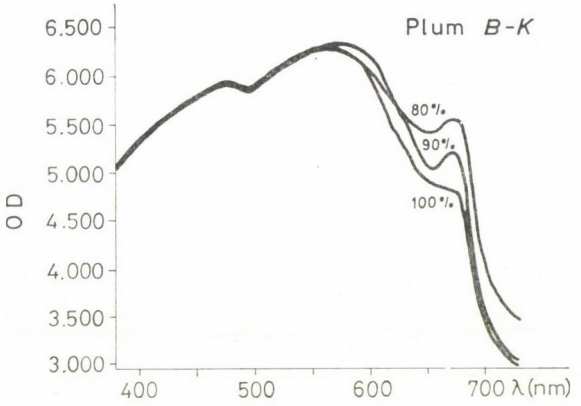

Fig. 6. Transmission OD spectra of plum samples (variety *B–K* grown in Zsámbék) for 80, 90 and 100% ripeness. Diameter of diaphragm 20 mm. Illumination parallel to fruit axis

and the OD values of fruit samples obtained from the studied wavelength range in 0.5 nm steps. Thereafter the apparatus plotted the correlation coefficients against wavelength as can be seen in Fig. 7 for sour-cherry. The instrument then determined, in a similar way, the second characteristic wavelength, considering the first as fixed.

The pairs of characteristic wavelengths for predicting the other quality parameters such as dry matter content, acid content, refraction were determined by the same method.

Table 1 summarizes the pairs of characteristic wavelengths, the coefficients of regression equations, the standard error of estimate of quality parameter values predicted from these regression equations and the multiple correlation coefficients for sour-cherry variety *P–141*.

Fig. 7. The spectrum of correlation coefficients which characterize the relationship between ripeness of sour-cherry (variety *P–141* grown in Budaörs) and the respective OD value measured at different given wavelengths

Table 1

Correlation between sour-cherry (variety P–141*) quality parameters and OD values measured at two characteristic wavelengths*

Equation form $Q_1 = k_0 + k_1 OD\lambda_1 + k_2 OD_2 \lambda_2$

	λ_1 (nm)	λ_2 (nm)	k_0	k_1	k_2	Standard error of estimate	Correlation coefficient
Ripeness	585	700	79.20	13.02	–27.23	2.89	0.972
Dry matter content	585	725	18.34	1.61	– 5.08	0.44	0.925
Acid content	588	717	3.66	–0.198	– 0.21	0.08	0.894
Refraction	585	610*	17.48	5.32	– 9.28	0.85	0.875

Equation form $Q_2 = k_3 + k_4 \frac{OD\lambda_3}{OD\lambda_4}$

	λ_3 (nm)	λ_4 (nm)	k_3	k_4	Standard error of estimate	Correlation coefficient
Ripeness	585	689	16.61	30.76	3.05	0.968
Dry matter content	690	586	27.48	–20.26	0.52	0.893
Acid content	522*	602	0.70	0.95	0.05	0.838
Refraction	618	586	36.76	–36.68	0.96	0.831

Data marked with asterisks (*) are wavelengths not sharply defined (here the spectra of correlation coefficients are flat) which means that similar results were obtained with OD values measured at other wavelengths

The unit for ripeness is percentage of full ripeness

The units for dry matter and acid content are weight %

Sugar content was measured refractometrically

Standard errors are expressed in the same units

In Table 2., the values of ripeness sensorically assessed (by RIFGOP's skilled expert) and the respective values obtained from the regression equation (Formula 1) as well as their differences are listed for the studied 60 sour-cherry samples.

Table 2

Detailed list of percentage of ripeness assessed sensorically and predicted by OD measurements for the 60 sour-cherry samples

Percentage of ripeness

No. of sample	Assessed	Predicted	Difference	No. of sample	Assessed	Predicted	Difference
1	60.0	66.3	−6.3	31	80.0	82.4	−2.4
2	60.0	60.4	−0.4	32	80.0	80.5	−0.5
3	60.0	65.4	−5.4	33	80.0	79.1	0.9
4	60.0	61.4	−1.4	34	80.0	77.5	2.5
5	60.0	58.9	1.1	35	80.0	78.6	1.4
6	70.0	64.1	5.9	36	92.5	92.4	0.1
7	70.0	70.4	−0.4	37	92.5	97.2	−4.7
8	70.0	68.6	1.4	38	92.5	92.7	−0.2
9	70.0	69.8	0.2	39	92.5	91.0	−1.5
10	70.0	69.4	0.6	40	92.5	91.1	1.4
11	80.0	82.6	−2.6	41	75.0	76.0	−1.0
12	80.0	78.3	1.7	42	75.0	77.6	−2.6
13	80.0	78.1	1.9	43	75.0	76.7	−1.7
14	80.0	74.3	5.7	44	75.0	77.5	−2.5
15	80.0	79.0	1.0	45	75.0	74.7	0.3
16	90.0	85.3	4.7	46	80.0	78.6	1.4
17	90.0	93.3	−3.3	47	80.0	84.1	−4.1
18	90.0	92.7	−2.7	48	80.0	79.1	0.9
19	90.0	92.9	−2.9	49	80.0	80.1	−0.1
20	90.0	94.7	−4.7	50	80.0	82.1	−2.1
21	62.5	65.2	−2.7	51	92.5	88.9	3.6
22	62.5	67.1	−4.6	52	92.5	96.0	−3.5
23	62.5	60.5	2.0	53	92.5	92.4	0.1
24	62.5	63.4	0.9	54	92.5	88.3	4.2
25	62.5	63.2	−0.7	55	92.5	92.4	0.1
26	70.0	69.6	0.4	56	100.0	96.3	3.7
27	70.0	69.8	0.2	57	100.0	95.6	4.4
28	70.0	72.2	−2.2	58	100.0	99.8	0.2
29	70.0	68.0	2.0	59	100.0	97.3	2.7
30	70.0	63.9	6.1	60	100.0	97.7	2.3

3. Conclusion

Our preliminary studies, performed on the four stone fruit species, cherry, sour-cherry, apricot and plum, have confirmed the statement well known from the literature that the ripeness of fruits is predictable on the basis of OD values measured at two different characteristic wavelengths of the transmission spectra.

Analysing data in Table 1 revealed that the two forms of the regression equations give practically the same results in predicting the quality parameters of stone fruits by measuring their OD values.

As can be seen in Table 1, the wavelength-pairs for predicting the different quality parameters are very close to one another. The OD values measured at one of the two wavelengths of each wavelength-pair are greatly influenced by the anthocyanin content. The correlation coefficients for predicting the ripeness are the best ones. Consequently, it is the ripeness (correlating well with the anthocyanin content) that is predicted on the basis of the anthocyanin content and the prediction of the other three quality parameters — which are, of course, not independent of ripeness — are indirect and therefore they may be incidental.

Similar characteristic wavelengts were found for plums and apricots in the studies of KRIVOSHIEV, but the varieties examined were different from those described here. So far no report has been found in the literature on cherries and sour-cherries.

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DETERMINATION OF THE QUALITY OF RAW TOMATO JUICE WITH AN OPTICAL RESEARCH COMPOSITION ANALYSER

L. HORVÁTH, and M. HORVÁTH-MOSONYI,

(Received: 2 February 1980; accepted: 25 March 1980)

The relationship between the optical properties of raw tomato juice and quality of the raw material was studied. The constituents of fresh juice made of partially ripe and fully ripe tomatoes were determinded by chemical methods.

The main constituents of the colouring matter of the two samples were: lycopene: 1.9 and 4.4 mg per 100 g fresh weight, β-carotene: 0.26 and 0.20 mg per 100 g fresh weight, total chlorophyll: less than 1 mg per 100 g fresh weight (in both samples).

By mixing the two tomato juices in different ratios, 33 different samples of different quality were made with determined constituents. The OD, ΔOD, and relative energy spectra were evaluated mathematically.

The best correlation between the actual and calculated percentages of mixing was found at 669 nm, by means of the ΔOD (669–620 nm) value. The best linear regression equation was found: computed % = $-32.7-2302$ ΔOD (669–620 nm), the figure of merit was 12.6, the standard error of estimate was 3.9 and the correlation coefficient was $r = 0.99$ [Figure of merit = Range of results/2 × ×standard error of estimate].

Summing up the results of the preliminary studies, it can be stated that the mixing rate of the samples composed of the two raw materials of different quality but known composition can be reliably computed on the basis of their spectra. Given the mixing ratio, the pigment content can be determined exactly.

The composition of tomatoes changes during ripening. We can measure certain components determining quality such as lycopene, β-carotene, total chlorophyll by traditional chemical analysis only in a complicated and time-consuming way.

It is in the interest of tomato production and processing to develop an instrument as well as a method by which the stage of ripeness and the composition pertaining to that stage is determined quickly and exactly in an objective way.

BIRTH and co-workers declared already in 1957: "In processing into tomato juice, puree, or as canned tomatoes, the interior color of the tomato is of greater importance than the external color". For qualifying interior colour, instrumentation techniques have been developed which measure and record the spectral transmittance of intact tomatoes.

BIRTH and NORRIS (1965) developed a two-filter difference meter for measuring interior spectral properties of foods and pigment in biological tissues.

Using a 4-filter photometer, WORTHINGTON (1974) reported that the light transmittance measured by the photometer, was effective for sorting fruits, based on stage of ripeness.

KRIVOSHIEV (1974) developed a two-filter tomato-sorting equipment on the basis of the transmittance properties of whole tomatoes.

WATADA and co-workers (1976) estimated the relationship between the absorbance properties and pigment composition of intact tomatoes.

JEN and co-workers (1977) studied the presence of phytochrome by *in vivo* measurements in two kinds of tomatoes on the basis of light absorbance technique.

Data collected over several years using the light-transmittance sorting technique of intact tomatoes and other fruits was published by KRIVOSHIEV and CHALUKOVA in 1980.

At the CENTRAL FOOD RESEARCH INSTITUTE in Budapest a research composition analyser, NEOTEC 6450 was installed in 1979. This paper deals with preliminary studies on raw tomato juice.

For spectral analysis, we need a great number of chemically analysed raw tomato juice samples of known composition. The computer built into the instrument determines the correlation between the composition and spectra of samples.

Chemical analysis was made at the food chemistry laboratory of the FACULTY OF ADVANCED PARAMEDICAL TRAINING AT THE INSTITUTE FOR POSTGRADUATE MEDICAL EDUCATION, DEPARTMENT OF DIETETICS, Budapest.

1. Materials and methods

To reduce chemical analytical work we initiated our research from samples of two different stages of ripeness. Partially ripe and ripe tomatoes were picked in January at *Szentes*, variety *Kecskeméti* 3 (*K* 3). After crushing the skin and seeds were removed by a screen of 1.7 mm mesh. After chemical analysis samples were mixed in various proportions at 10% intervals *i.e.* 1 to 9, 2 to 8, 3 to 7 *etc.*

This resulted in 11 samples of known composition. Optical transmission measurements were carried out in the composition analyser, NEOTEC 6450 of the Neotec Instruments Incorporated (USA) repeated in holders of 10 mm thickness with 3 different samples of each mixture. Consequently we had 33 spectra for spectral analysis. The OD, ΔOD and relative energy (R) spectra were mathematically evaluated.

The determination of carotenoids was carried out by thin-layer chromatography as recommended by the RESEARCH INSTITUTE OF THE CANNING and PAPRIKA INDUSTRIES in Budapest (unpublished data).

The absolute dry matter content was determined at first by drying the samples at 105 ± 2 °C, then a sample equivalent to 1 g absolute dry matter was measured in an *Erlenmeyer* flask and was allowed to stand with methanol with occasional shaking for 15 min. After this, it was filtered through a *Büchner* funnel. The residue was shaken by a machine with a mixture of hexane—acetone (1 : 1) for 10 min, then the acetone was washed out with water. The process of extraction was repeated once more with a hexane—acetone (1 : 1) mixture and the hexane solution was dried over anhydrous sodium sulfate. The pigment content of the methanol filtrate was transferred to petroleum ether and it was also dried over anhydrous sodium sulfate.

The water-free hexane and petroleum ether solutions were combined and evaporated in a water bath at 50 °C in vacuum and in N_2. The dried residue was dissolved in benzene and was then chromatographed.

The separation of the pigments was carried out on an activated *Kieselgel* G layer with two different solvent mixtures: we initiated the development of the chromatogram with a mixture of petroleum ether—benzene—acetone (40 : 10 : 5) till the distance of 3 cm from the starting line, then the chromatogram was dried in air and development was made by a petroleum ether—benzene (50 : 5) mixture till the top of the plate.

The bands corresponding to lycopene and β-carotene were scraped off from the plate, the pigments were dissolved in acetone and were separated from the material of the layer by filtering on a glass funnel using suction. The solutions were made up to volume with acetone and the optical density of the acetone solutions was measured with a SPEKTROMOM 201 spectrophotometer, the lycopene at 472, the β-carotene at 454 nm corresponding to their absorbance of maxima.

For calculating the β-carotene and lycopene contents, extinction coefficients (optical densities) determined from the calibration line by the RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRIES in Budapest were used. These values were: 0.660 in the case of β-carotene and 0.593 in the case of lycopene.

For determining the chlorophyll content, the modified AOAC method published by SWEENEY and MARTIN (1961) was used. The sample was extracted with acetone several times, the pigment of the combined acetone solutions was transferred to petroleum ether, and washed by repeated bubbling through water, then dried over anhydrous sodium sulfate. Following this, we tried to separate the pigments by chromatography on a column of powdered sugar on the basis of the recommended method but this was not satisfactorily evaluable because of the low concentration of chlorophyll. A thin-layer chromatogram was also made on a silicagel–cellulose (2 : 1) layer without impregnation with petroleum ether–benzene–pure alcohol (5 : 1 : 0.5) mixture.

On the thin-layer chromatograms for carotenoids and for chlorophylls of the partially ripe tomato we found two spots, a yellow and a green one which could not be found on the chromatograms of the ripe tomato. Comparing them to the chromatogram of an extract made of green leaves we established that they were the derivatives of chlorophyll. However the amount of these could not be determined in any case.

Consequently it can be stated that in the samples examined, the amount of chlorophyll cannot be determined by traditional chemical analysis. Given the data for lycopene and β-carotene and taking into account the data published by WATADA and co-workers (1976), the chlorophyll content of our samples must be less than 1 μg per g fresh sample.

The water-soluble dry matter of the samples was determined by a *Zeiss-Abbe* refraktometer at 20 °C.

The definitions of the symbols used in this paper are:

optical density: $\mathrm{OD}(\lambda) = \log [I_0(\lambda)/I(\lambda)]$

relative energy: $R = I_0(\lambda)/I(\lambda)$

where: $I_0(\lambda)$is the light intensity measured for the reference at λ

$I(\lambda)$ is the light intensity measured for the sample at λ

prepared sample data: R_p

measured data: R_m

calculated data: R_c (calculated on the basis of the regression function determined from the measured data)

$$\text{standard error of estimate} = \sqrt{\frac{\sum_{i=1}^{n} (R_{m_i} - R_{c_i})}{n-2}}$$

$$\text{figure of merit} = \frac{\text{range of results}}{2 \times \text{standard error of estimate}}$$

number of samples: n

2. Results and discussion

Some chemical characteristics of the partially ripe and fully ripe samples are shown in Table 1.

Pigment quantities refer to fresh material.

Table 1

Some chemical characteristics of the tomato samples examined

Sample	Soluble dry matter (refr. %)	Total dry matter (%)	Lycopene (mg 100 g^{-1} fw*)	β-carotene (mg 100 g^{-1} fw*)	Total chlorophyll (mg 100 g^{-1} fw*)
Partially ripe	2.6 ± 0.05	4.2 ± 0.1	1.9 ± 0.1	0.26 ± 0.08	< 0.1
Ripe	2.6 ± 0.05	4.3 ± 0.1	4.4 ± 0.3	0.20 ± 0.07	< 0.1

* fresh weight

The transmission OD spectra of samples were taken in the visible range of 380–730 nm. First we measured the OD spectra. In Fig. 1 the OD spectra of partially ripe samples containing 0, 20, 50, 80 and 100%, respectively, of ripe

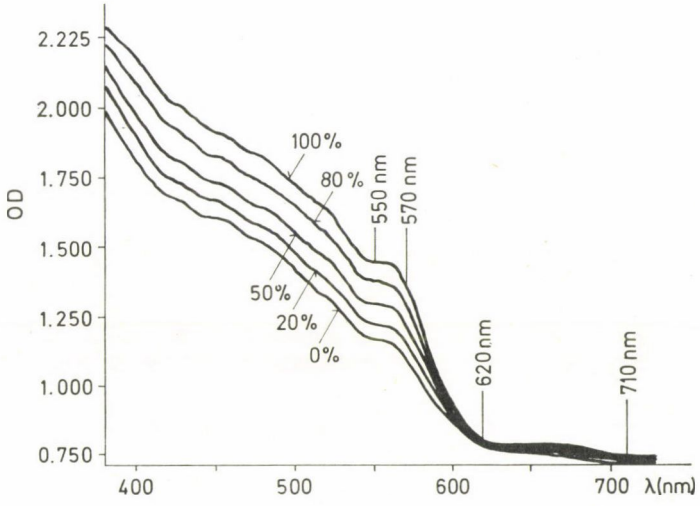

Fig. 1. The OD spectra of mixtures of partially ripe and fully ripe tomato samples in the visible range. (Inscriptions indicate the percentage of fully ripe tomatoes in the mixture)

tomato can be seen. The spectra have a flat portion around 550 nm and cross one another at 620 nm.

In the Figure, the 550, 570 and 710 nm wavelengths are marked. WATADA and co-workers (1976) determined the β-carotene content from the absorbance difference ΔA (550–580 nm), the lycopene content from ΔA (570–780 nm) and the chlorophyll content from ΔA (710–780 nm).

Figure 2 shows the enlarged section of the OD spectrum between 620 and 730 nm.

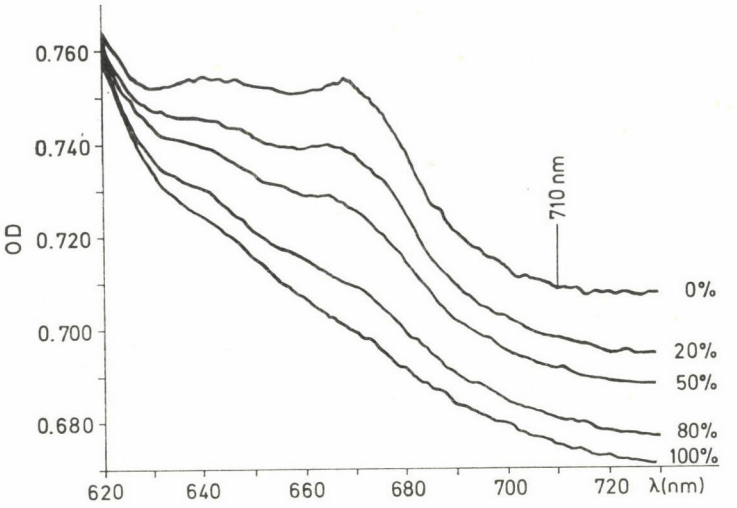

Fig. 2. The OD spectra of mixtures of partially ripe and fully ripe tomato samples in the range of 620–730 nm. (Inscriptions indicate the percentage of fully ripe tomatoes in the mixture.)

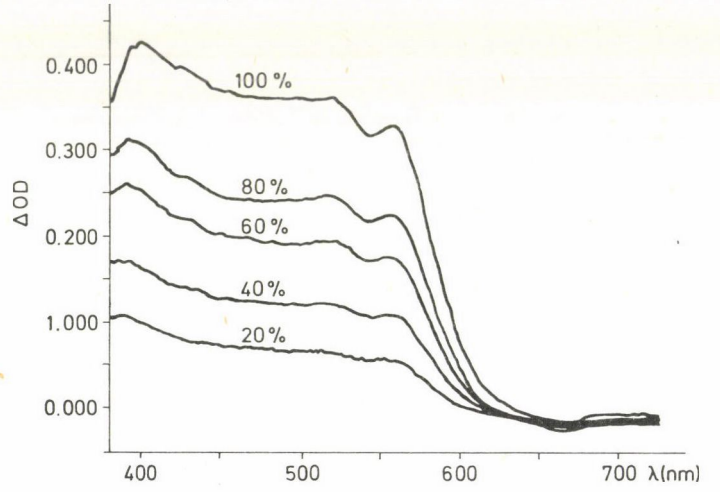

Fig. 3. The ΔOD spectra of mixtures of partially ripe and fully ripe tomato samples related to the partially ripe sample. (Inscriptions indicate the percentage of fully ripe tomatoes in the mixture)

Maxima, characteristic of chlorophyll, can be seen around 670 nm in the partially ripe sample. These maxima are hardly noticeable in the case of the ripe sample.

The mathematical programs of NEOTEC 6450 open up the possibility of presenting the ΔOD spectrum seen in Fig. 3, where the 0% partially ripe sample spectra served as the basis for comparison.

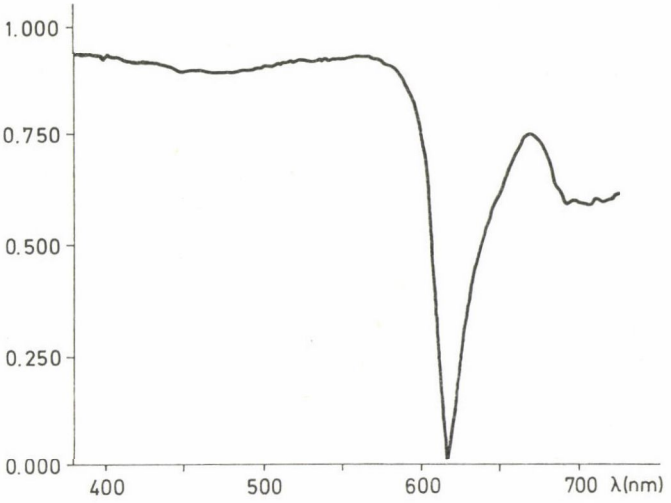

Fig. 4. Coefficients of correlation (r) between the R_p (prepared-sample data) and R_c (fully ripe %) values of tomato samples determined from the ΔOD spectra

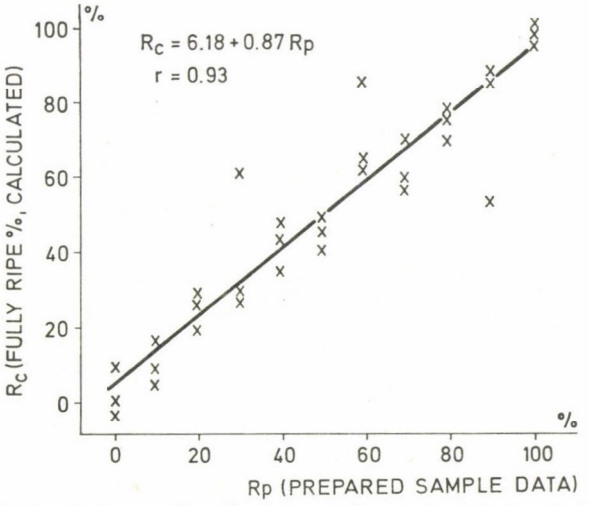

Fig. 5. The correlation between the R_p (prepared-sample data) and R_c (fully ripe %), values of tomato samples at 396 nm

The relative spectra possess maxima at around 400, 420, 520 and 560 nm, respectively which are hardly noticeable in the case of ripe samples.

The next step was to determine the relationship between OD spectra and mixtures of different ratios. By means of a regression program the equipment has determined the coefficients of the R_c (fully ripe %) $= K_0 + K_1 \cdot$ OD shaped equation as well as the correlation coefficients at each wavelength. Thereafter the maximum of the correlation coefficients was determined and the optimal wavelength and coefficients were given for the maximum.

Figure 4 shows the correlation coefficients determined at different wavelengths.

The coefficients have a maximum at 391 nm where the correlation factor $r = 0.93$, the standard error of estimate is 11.5% and the figure of merit is 4.4. The function has a 0 point at the crossing point of spectra at 620 nm.

Figure 5 shows the correlation between the prepared-sample and calculated values of the mixing ratio determined at 396 nm by means of the regression function:

$$R_c(\text{fully ripe } \%) = -455.9 + 254.9\ OD_{396} \qquad (r = 0.94)$$

It can be seen that the correlation is not too close. The relation between the prepared-sample and measured data can be improved with different mathematical operations. In the first step, the OD values determined at every 0.5 nm of the spectrum were substituted by an average of 6 neighbouring values. We do not have to take into consideration any loss of information with OD values determined, in such a way, at every 3 nm.

Thereafter we subtracted the OD value of the 620 nm crossing point from the modified spectrum. With this, we got the spectra expressed in OD and these already cross one another not around but exactly at 620 nm.

The correlation function determined on the basis of the modified spectra can be seen in Fig. 6.

It is striking to see the difference between the correlation functions determined on the basis of unmodified and of corrected spectra. Their common feature is a minimum at 620 nm. The maximum of the function is at 669 nm.

Figure 7 shows the regression function determined at 669 nm as well as the percentage values computed by means of the function.

$$R_c(\text{fully ripe}\%) = 32.7\text{–}2302\ \Delta OD_{669} \qquad (r = 0.99)$$

The fit of the measured points to the regression line can well be seen. As a result of spectrum correction, the correlation coefficient rose from 0.93 to 0.99, the figure of merit from 4.4 to 12.6 and the standard error of estimate decreased from 11.5 to 4.0%.

We performed further spectrum corrections as well. We also determined the relative energy spectra compared to the 620 nm point. The optimal wavelength determined from the relative energy spectra was also 669 nm with a correlation coefficient of $r = 0.99$ and a standard error of estimate of 3.9%.

We must mention that we also performed the examinations relating OD values to the flat portion of the spectrum at 550 nm.

The optimal wavelength proved to be 557 nm with a correlation factor of $r = 0.99$ and standard error of estimate of 6.6%. These latter corrections brought about no further gain.

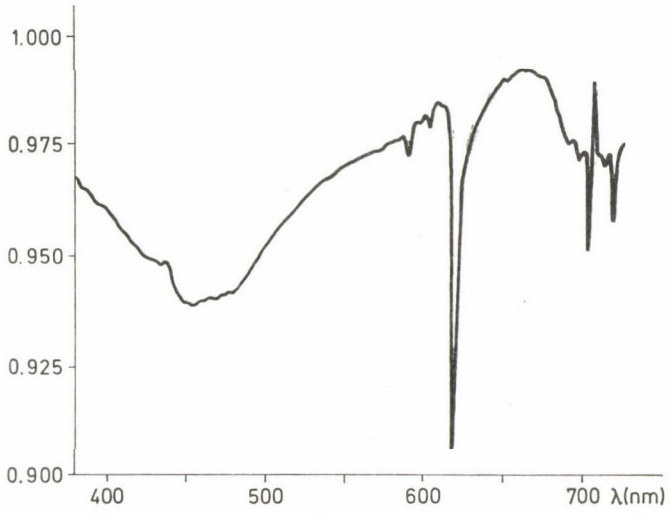

Fig. 6. Coefficients of correlation (r) between the R_p (prepared-sample data) and R_c (fully ripe %) values of tomato samples determined from the ΔOD spectra

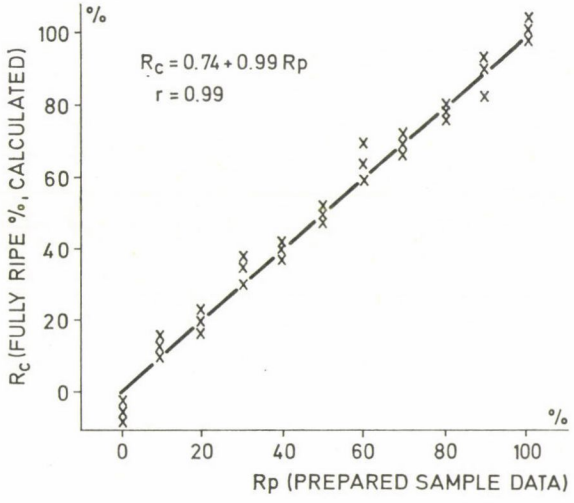

Fig. 7. The correlation between the R_p (prepared-sample data) and R_c (fully ripe %) values of tomato samples at 669 nm

The NEOTEC 6450 research composition analyser possesses quite a number of further possibilities. We are in the process of identifying newer corrections until we manage to find the most suitable correction.

Summing up the results of the preliminary studies, it can be stated that the mixing rate of the samples composed of the two raw materials of different

quality but known composition can be reliably computed on the basis of their spectra. Given the mixing rate the pigment content can already be determined exactly.

The results published here are part of a preliminary experiment the final aim of which is not only to determine the composition of raw tomato juice but also to provide an objective and quick method for the determination of the composition of whole tomato.

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BIOLOGICAL VALUE OF FUNGAL PROTEINS BASED ON THEIR AMINO ACID COMPOSITION

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(Received: 22 February 1980; accepted: 8 May 1980)

Fourteen different strains of *Mucor*, *Actinomucor* and *Rhizopus* species were cultivated on waste bread.

Amino acid composition of their mycelia was determined and the egg ratios calculated.

The evaluation of the nutritional value of the tested biomasses has been carried out by methods which relate amino acid composition of the tested protein to that of whole egg, (MITCHELL & BLOCK, 1946; OSER, 1951; KORPÁCZY *et al.*, 1961; SPEIDEL & BENNETT, 1972) and a method which uses the amino acids of a protein mixture (potato and egg) as reference amino acids (MØRUP & OLESEN, 1976). This mixture has the highest biological value for man in a 10-year N-balance experiment (KOFRÁNYI *et al.*, 1970).

In the course of the comparison of the above methods, there was a close correlation between the method of *Oser* and *Speidel & Bennett*. Good correlation was found also between the following methods: *Oser* vs. *Mitchell & Block; Speidel & Bennett* vs. *Mitchell & Block* but a loose correlation between the methods of *Mørup & Olesen* vs. *Mitchell & Block; Mørup & Olesen* vs. *Speidel & Bennett;* and *Mørup & Olesen* vs. *Korpáczy et al.*, resp. No correlation was detectable between the following methods: *Mørup & Olesen* vs. *Oser; Mitchell & Block* vs. *Korpáczy et al.;* and *Speidel & Bennett* vs. *Korpáczy et al.*

The need for additional sources of food, especially of protein, opened up new horizons of the fermentation industry, developing a new branch, the production of single cell protein. The fermentation of single cells was very soon followed by the fermentation of filamentous microorganisms (GRAY, 1965). As the latter can utilize crude raw materials, protein production utilizing various wastes for human foods directly (GRAY & ABOUD-EL-SEOUD, 1966; SPICER, 1973; CHRISTIAS *et al.*, 1975) or indirectly as animal feed (CHURCH *et al.*, 1973; ADER & PLASKETT, 1975; IMRIE, 1975) has soon become the subject matter of intensive research efforts. CHRISTIAS and co-workers (1975) found the quality of some fungal proteins superior to that of yeast protein because of their lower nucleic acid content, better structure and their easier filterability.

The cultivation of many kinds of microorganisms is necessary to select the best strain for the production of microbial proteins. Selection of strains according to mycelial yield and protein content is not satisfactory, the nutritional value of the individual biomasses must be determined, too.

The evaluation of nutritive value by animal feeding test involves long-term multigeneration experiments. These are expensive, need a long time and many parallel tests to eliminate factors disturbing the evaluation.

Chemical methods are therefore indispensable for the selection of suitable strains.

In our work, various *Mucor*, *Actinomucor* and *Rhizopus* strains were cultivated on waste bread to produce protein for human food. For the prediction of their nutritional value, their amino acid content was determined and their biological value was calculated by five different methods.

1. Materials and methods

1.1. Microorganisms

Eight *Rhizopus*, three *Mucor* and three *Actinomucor* strains (from the strain collection of the RESEARCH INSTITUTE FOR VITICULTURE AND OENOLOGY, Budapest) were used. The names and catalogue numbers of the investigated microorganisms are given in Table 1.

Table 1

The signs and names of the various strains

Sign of the strain	Name of the fungus
202	*Rhizopus nigricans*
206	*Mucor racemosus*
207	*Actinomucor repens*
208	*Actinomucor repens*
209	*Rhizopus sp.*
211	*Rhizopus circinans*
216	*Rhizopus arrhizus*
217	*Actinomucor repens*
219	*Mucor sp.*
221	*Rhizopus cohnii*
222	*Rhizopus cohnii*
223	*Rhizopus microsporus*
224	*Mucor mucedo*
225	*Rhizopus arrhizus*

1.2. Maintenance of strains

Stock cultures were maintained on malt agar slants.

1.3. Nutrient media

The composition of the inoculation medium was the same as that given in a previous paper (ZETELAKI-HORVÁTH *et al.* 1975).

A fermentation medium of the following composition was used: ground bread 4%, corn-steep liquor 10 g, $(NH_4)_2SO_4$ 10 g, $ZnSO_4$ 0.25 g, $MnSO_4$ 0.10 g, KH_2PO_4 1.0 g. The volume of medium was made up to 1 000 cm^3 with tap water; the pH was adjusted to 4.0.

1.4. Method of cultivation

The method of cultivation and the preparation of the mycelia were also given previously (ZETELAKI-HORVÁTH *et al.*, 1975).

1.5. Protein determination

The protein content of mycelia was determined by the biuret method as modified for protein determination of whole cells by HERBERT and co-workers (1971).

1.6. Preparation of the mycelia for amino acid analysis

Fifty mg of dried, ground mycelium were hydrolyzed with 5 cm^3 of 6 *N* HCl at 110 °C for 24 hours in 10-cm^3 ampoules. After hydrolysis, the samples were filtered on a glass filter and dried in a vacuum evaporator (KUTESZ, Hungary) at 45 °C. The residue was washed twice with distilled water and evaporated to dryness, then it was taken up into 10 cm^3 of distilled water.

1.7. Amino acid analysis

A Hd 1 200 E type, two-column amino acid analyser (Závad SNP, Ziar nad Hronom, Czechoslovakia) was used for the quantitative determination of amino acid.

1.8. Determination of tryptophan

Tryptophan was determined by the method of FOLIN and CIOCALTEU (1927).

1.9. Calculation of biological value

Estimation of the biological value of the fungus mycelium was carried out by the following methods:

1.9.1. Chemical score. According to the "chemical score" *(CS)* concept of MITCHELL and BLOCK (1946), the biological value of a protein can be estimated from its maximum percentage deficit in an essential amino acid (D) by the equation:

$$CS = 102 - 0.634\,D \tag{1}$$

1.9.2. Essential amino acid index. The essential amino acid index *(EAA*, calculated from ten essential amino acids) is the geometric mean of the ten nutritional egg ratios (OSER, 1951), computed logarithmically:

$$\log EAA = \frac{1}{10}\left(\log\frac{100a}{a_e} + \log\frac{100b}{b_e} + \ldots + \log\frac{100j}{j_e}\right) \tag{2}$$

where a, $b \ldots j$ represent the percentages of each of the ten essential amino acids in the food protein ($N \times 6.25$) and a_e, b_e, $\ldots$ j_e stand for the percentages of the respective amino acids in whole egg protein.

1.9.3. Modified essential amino acid index. SPEIDEL and BENNETT (1972) also used the essential amino acid index for the calculation of biological value of proteins, but they complemented this with a factor of digestibility, according to the equation:

$$SB = 1.09(EAA) - 11.73 \tag{3}$$

1.9.4. Biological value based on all amino acids. KORPÁCZY and co-workers (1961) calculated biological value (K) on the basis of the total amino acid content (essential and non-essential) of proteins by means of the following equation:

$$K = 0.75A + 0.25B \tag{4}$$

where:

A = biological value of essential amino acids calculated by *Oser*'s method

B = biological value of non-essential amino acids, also calculated by *Oser*'s method.

They have modified the calculation of OSER, (1951) when the concentration of the compared amino acids is higher than that in whole egg. In this case, they use the following quotient:

$$\frac{b_t}{b_x} \cdot 100 \tag{5}$$

where:

b_x = concentration of that amino acid (in the investigated protein) which is higher than the concentration of the same amino acid in whole egg protein

b_t = concentration of the same amino acid in whole egg protein

1.9.5. Biological value based on human feeding experiments. MØRUP and OLESEN (1976) developed an equation for the prediction of biological value (PV) from the data of N-balance experiments of KOFRÁNYI and co-workers (1970) carried out on man. [As reference amino acids (mg total essential) the data of a potato—egg mixture were used: ile: 110; leu:179, lys: 141; arom. (phe + tyr): 212; sulp. (meth + cys): 89; thr: 99; try: 30; val: 140.]

The equation for the predicted biological value is the following:

$$PV = 10^{2.15} \times q_{lys}^{0.41} \times q_{arom}^{0.60} \times q_{sulp}^{0.77} \times q_{thr}^{2.4} \times q_{try}^{0.21} \qquad (6)$$

2. Results

2.1. Amino acid spectra of the strains

The amino acid composition of the investigated strains showed great differences not only among the various species but also among strains of the same species (Tables 2 and 3).

As can be seen in Table 2, the concentration of the majority of amino acids was highest in the mycelia of the *Rhizopus* strains. Phenylalanine, arginine, valine, leucine, iso-leucine, methionine and threonine were present in highest concentration in the mycelium of *Rhizopus cohnii*, Strain No. 221, while the histidine and lysine content was highest in the mycelia of Strain No. 225.

Comparing the average values of amino acids of the investigated strains, the histidine and arginine content was highest (3.79 and 5.0%) in the mycelia of *Rhizopus* strains, while the average value of valine, leucine, iso-leucine and threonine was highest (4.74, 6.64, 3.54 and 3.59% resp.) in the mycelia of *Mucor* strains.

2.2. Egg ratio of essential amino acids

Since amino acid composition of the whole egg is considered to represent a nutritional value of 100%, the egg ratio for each strain has been calculated.

In contrast to *Oser*'s procedure, egg ratios of the investigated strains were given in their real values, namely the egg ratios of the amino acids higher than that of the whole egg, has not been taken as 100%.

The percentage ratios of amino acids in mycelial protein of the individual strains relative to their content in whole egg protein are given in Tables 4 and 5 with the essential amino acid content of the whole egg.

According to their essential amino acid spectra and egg ratios, *Rhizopus cohnii* strains (Nos. 221 and 222) proved to be the best (Table 4), while, of

Table 2

Amino acid composition of the mycelia of various Rhizopus *strains. Figures indicate averages* ($\bar{x}$) *and standard deviations* (s) *of the amino acid content* (%) *of the protein (three parallel determinations; cultivation on a shaker at 330 rpm; carbon source : ground bread).*

Amino acid	*Rhizopus* strains															
	202		209		211		216		221		222		223		225	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	x	$\pm s$	$\bar{x}$	$\pm s$
Lysine	4.15	1.04	5.52	1.49	5.02	1.67	6.20	0.61	9.81	1.91	8.55	1.31	5.01	0.96	12.00	2.39
Isoleucine	2.77	0.92	3.10	0.72	2.18	0.25	3.28	0.32	4.92	0.84	3.93	0.35	2.69	0.09	2.55	0.52
Valine	3.23	0.64	3.50	0.69	2.56	0.34	2.91	0.50	6.67	1.71	6.03	1.05	3.03	0.25	3.27	0.21
Arginine	4.20	0.84	3.91	0.68	3.81	0.28	4.95	0.37	7.92	1.55	6.64	1.14	4.09	0.80	4.51	0.67
Methionine + cystine	1.06	0.09	1.40	0.11	0.77	0.14	1.33	0.13	1.98	0.45	1.35	0.14	0.96	0.25	1.66	0.25
Threonine	2.26	0.36	3.12	0.67	2.14	0.71	3.22	0.57	6.34	0.75	5.21	1.45	2.73	0.81	2.90	0.45
Leucine	5.39	1.32	5.74	1.23	3.70	0.55	4.31	0.61	8.78	1.33	6.32	1.85	4.24	0.58	4.82	1.09
Phenylalanine	3.90	0.48	11.42	1.95	3.18	0.54	2.88	0.28	15.05	2.12	10.66	2.01	3.18	0.76	2.75	0.32
Histidine	2.73	0.36	3.05	0.72	2.59	0.69	3.07	0.46	4.04	0.93	3.61	0.36	2.59	0.39	8.67	2.01
Trytophan	1.91	0.23	1.80	0.51	2.00	0.18	1.87	0.19	2.02	0.35	1.79	0.21	2.12	0.21	1.55	0.25
Aspartic acid	4.55	1.41	5.40	1.46	3.67	0.69	6.74	0.66	9.10	1.04	7.71	1.95	5.19	0.80	6.04	0.67
Serine	3.52	0.68	3.80	0.67	2.32	0.82	3.36	0.40	6.83	0.52	5.53	1.20	2.86	0.58	3.53	0.47
Glutamic acid	19.31	1.24	14.65	2.40	6.14	2.11	7.85	1.14	16.98	2.06	16.35	2.18	8.65	1.36	9.22	1.33
Proline	6.92	0.69	7.25	1.31	2.55	0.44	1.45	0.35	6.32	1.00	5.39	1.46	2.93	0.64	3.69	0.29
Glycine	2.66	0.80	2.83	0.70	1.99	0.36	2.92	0.45	5.42	0.49	4.52	1.35	2.59	0.86	2.98	0.25
Alanine	2.77	1.11	3.48	0.44	2.48	0.34	3.07	0.50	7.10	1.53	5.65	1.22	3.46	0.35	3.42	0.91
Tyrosine	2.43	0.16	2.20	0.44	2.05	0.19	2.75	0.25	3.79	0.65	2.96	0.43	1.88	0.18	2.11	0.40

Table 3

Amino acid composition of mycelia of various Mucor *and* Actinomucor *strains. Figures indicate averages ($\bar{x}$) and standard deviations (s) of the amino acid content (%) of the protein (three parallel determinations; cultivation on a shaker at 330 rpm: carbon source : ground bread).*

Amino acids	*Mucor*						*Actinomucor*					
	206		219		224		207		208		217	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
Lysine	6.62	1.48	4.96	1.34	6.75	1.85	3.92	0.32	7.10	1.49	6.39	1.37
Isoleucine	3.98	0.64	3.24	1.04	3.39	0.61	3.17	0.52	2.98	0.82	3.61	1.07
Valine	4.66	1.28	5.37	0.74	4.18	0.93	3.26	0.61	4.02	0.69	2.75	0.58
Arginine	5.23	1.13	4.17	0.77	4.57	0.89	2.57	0.48	4.56	0.68	5.30	1.55
Methionine + cystine	1.19	0.38	1.18	0.24	0.98	0.19	0.18	0.08	0.70	0.14	0.90	0.21
Threonine	4.19	1.62	3.14	0.47	3.44	0.34	2.60	0.25	3.50	0.67	2.99	0.56
Leucine	7.19	1.25	5.50	1.31	7.24	1.21	5.07	0.95	5.91	1.21	5.63	1.07
Phenylalanine	7.92	1.35	3.51	0.92	3.91	0.82	2.71	0.51	3.89	0.96	4.16	1.06
Histidine	2.81	0.82	2.87	0.92	3.76	0.87	2.80	0.50	3.36	0.72	3.49	0.51
Tryptophan	1.66	0.28	1.47	0.25	1.67	0.31	1.41	0.25	1.54	0.34	1.91	0.28
Aspartic acid	7.84	1.95	6.30	1.86	7.58	1.79	5.31	1.31	7.39	1.46	6.40	1.20
Serine	4.61	1.37	3.64	1.38	4.23	1.07	2.76	0.61	3.69	0.67	3.69	0.93
Glutamic acid	16.04	1.89	14.94	1.66	11.89	2.30	8.86	1.90	9.07	1.40	14.74	2.38
Proline	13.49	1.34	5.96	1.82	8.60	2.06	2.47	0.35	7.15	0.81	4.04	1.07
Glycine	2.59	0.51	3.06	0.32	3.36	0.85	3.06	0.61	3.08	0.70	2.85	0.67
Alanine	4.25	0.77	3.71	0.67	3.86	0.92	3.52	0.55	4.08	0.43	3.52	0.64
Tyrosine	3.77	0.90	2.68	0.24	3.19	0.79	1.68	0.23	2.99		2.92	0.46

Table 4

Essential amino acids in protein of whole egg and egg ratio of mycelial protein of the various Rhizopus *strains*

Amino acids	Whole egg	Egg ratio							
		202	209	211	216	221	222	223	225
Lysine	7.0	59.29	18.86	71.71	88.57	140.14	122.14	71.57	171.43*
Tryptophan	1.5	127.33	120.00	133.33	124.66	134.66*	119.33	141.33	103.33
Isoleucine	7.7	35.97	40.26	28.31	41.95	63.90*	51.04	34.94	33.12
Valine	7.2	44.86	48.61	35.56	40.42	92.64*	83.75	42.08	45.42
Arginine	6.6	63.64	59.24	57.73	75.00	120.00*	100.61	61.97	68.33
Methionine and cystine	6.4	16.56	21.88	12.03	20.78	30.94*	21.09	15.00	25.94
Threonine	4.3	52.56	72.56	49.77	74.88	147.44*	121.16	63.49	67.44
Leucine	9.2	58.59	62.39	40.22	46.85	95.43*	68.70	46.09	52.39
Phenylalanine	6.3	61.90	181.27	50.48	45.71	238.89*	169.21	50.48	43.65
Histidine	2.4	113.75	127.08	107.92	127.92	168.33	150.42	107.92	361.25*

* highest value of the strains
– second best result

Table 5

Essential amino acid composition of whole egg and egg ratio of mycelial protein of the various Mucor *and* Actinomucor *strains*

Amino acids	Whole egg (%)	Egg ratio					
		Mucor			*Actinomucor*		
		206	219	224	207	208	217
Lysine	7.0	94.57	70.86	96.14	56.00	101.43*	91.29
Tryptophan	1.5	110.66	98.00	111.33	94.00	102.66	127.33*
Isoleucine	7.7	51.69*	42.08	44.03	41.17	38.70	46.88
Valine	7.2	64.72	74.58*	58.06	45.28	55.83	38.19
Arginine	6.6	79.24	63.18	69.24	38.94	69.09	80.30*
Methionine and cystine	6.4	18.75*	18.44	15.31	2.81	10.94	14.06
Threonine	4.3	97.44*	73.02	80.00	60.70	81.40	69.53
Leucine	9.2	78.15	59.78	78.70*	55.11	64.24	61.20
Phenylalanine	6.3	125.71*	55.71	62.06	43.02	61.75	66.03
Histidine	2.4	117.08	119.58	156.67*	116.70	140.00	145.42

* highest value of the strains
– second best result

the *Mucor* and *Actinomucor* strains, No. 206 (*M. racemosus*) gave the best results (Table 5).

It is apparent in the above Tables that methionine proved to be the amino acid in short supply, for all the investigated strains. The lysine content of three *Rhizopus* strains (Nos. 225, 221 and 222) was considerably higher than that of the whole egg. The egg ratios of histidine and tryptophan proved to be higher than 100% in the case of all but two strains tested (*Mucor* 219 and *Actinomucor* 207), having egg ratios for tryptophan of 98 , 94%, resp.

2.3. Biological value

It was attempted to compare the nutritive values of the biomass of the investigated *Rhizopus*, *Mucor* and *Actinomucor* strains, according to their biological value, calculated from the amino acid composition of mycelial proteins. Following the different methods, different amino acids (deficient, essential or total) were used for the calculations.

Biological values of the investigated *Rhizopus* strains are given in Table 6, while those of *Mucor* and *Actinomucor* strains in Table 7.

It is apparent from the tables that, in the majority of the investigated strains, highest values were obtained by the method of KORPÁCZY and co-workers (1961), as a consequence of the consideration of all amino acids, and the lowest by the method of MØRUP and OLESEN (1976). The latter was based on the results of feeding experiments (KOFRÁNYI *et al.*, 1970) carried out on adult humans.

Comparing the investigated *Rhizopus*, *Mucor* and *Actinomucor* strains, *Rhizopus cohnii* (No. 221) had the highest biological value according to the five methods applied. Only two methods (KORPÁCZY *et al.*, 1961; and MØRUP & OLESEN, 1976) gave a slightly higher biological value in the case of Strain No. 216 than in that of No. 221 (Table 6).

Table 6

Biological value of mycelial protein of the investigated Rhizopus *strains determined by different methods*

Method	*Rhizopus* strains							
	202	209	211	216	221	222	223	225
Oser	47.49	59.76	41.52	51.60	93.04	76.13	46.11	60.56
Korpáczy et al.	58.68	64.38	64.50	67.26	61.40	63.96	64.89	61.70
Mitchell & Block	54.99	60.25	50.51	59.19	69.22	59.48	53.44	64.27
Speidel & Bennett	40.03	53.41	33.53	44.52	89.68	71.25	38.53	54.28
Mørup & Olesen	40.98	40.03	39.29	43.23	43.01	31.64	40.92	31.68

– highest value of the investigated strains

Table 7

Biological value of mycelial protein of the investigated Mucor- *and* Actinomucor *strains*

Method	*Mucor*			*Actinomucor*		
	206	219	224	207	208	217
Oser	65.54	53.98	59.05	37.38	53.95	53.99
Korpáczy et al.	64.86	64.79	64.86	59.38	64.44	62.96
Mitchell & Block	57.15	56.85	53.75	41.33	49.42	52.52
Speidel & Bennett	59.71	77.11	52.63	29.01	47.07	47.11
Mørup & Olesen	40.98	54.95	40.15	19.23	29.64	38.80

– highest value of the investigated strains

Among the *Mucor* and *Actinomucor* strains, *Mucor racemosus* (No. 206) had the highest biological value according to four methods (Table 7), only the use of the method of MØRUP & OLESEN (1976) resulted in a higher biological value in the case of the strain No. 219.

The use of the methods of *Oser* and of *Speidel & Bennett* gave the highest value in the case of *Rhizopus* Strains Nos. 221 and 222 while the method of *Mitchell & Block* in the case of *Rhizopus* Strains Nos. 221 and 225. The

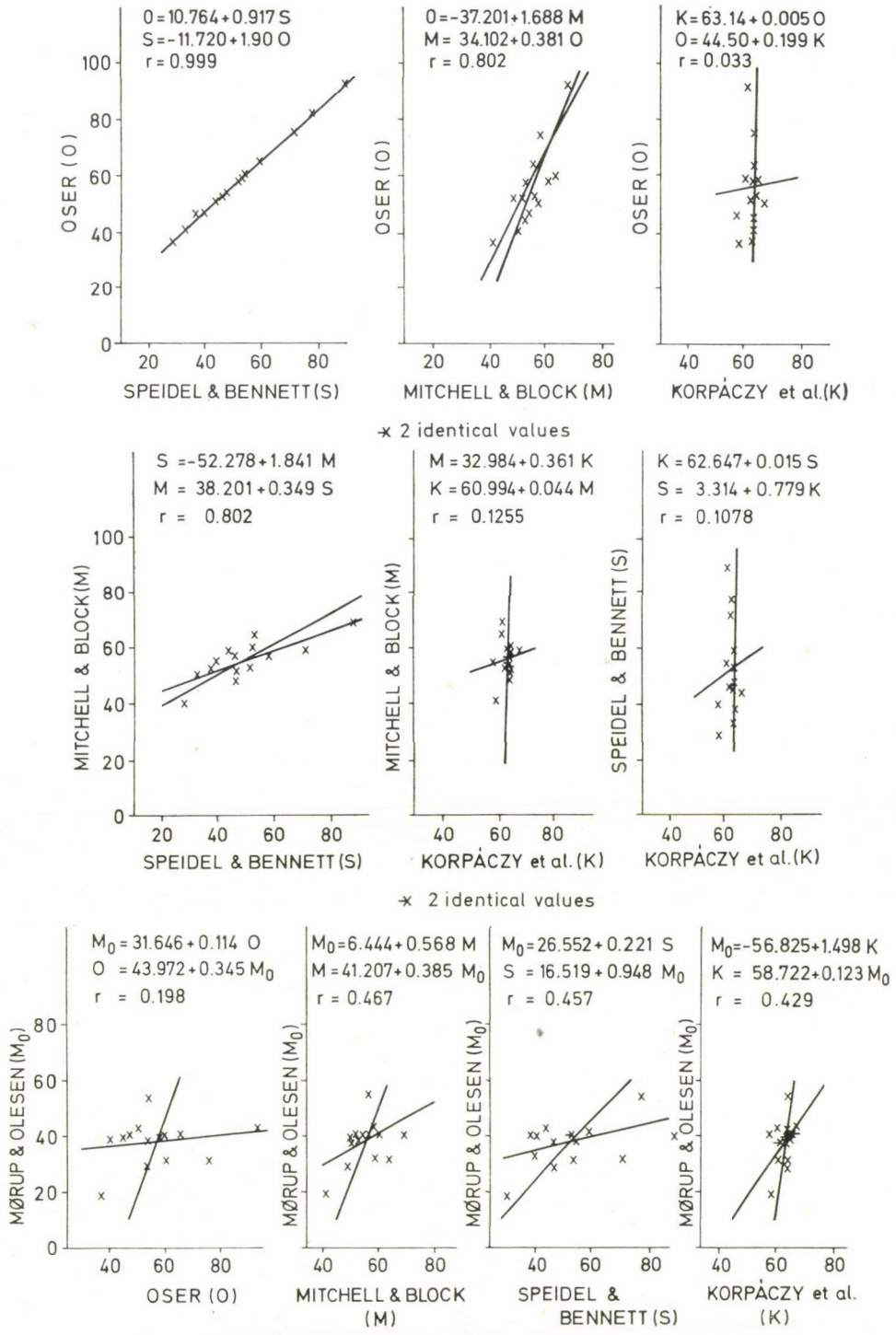

Fig. 1. Correlation between biological values of the various methods

biological value calculated by the method of *Speidel* and *Bennett* varied in correlation with the biological value of *Oser* but was always lower to a certain extent. The biological value of Strain No. 219 was the highest among *Mucor* and *Actinomucor* strains and No. 216 among *Rhizopus* strains when the method of MØRUP and OLESEN (1976) was used.

2.4. Correlation between biological values obtained by various methods

Very close and positive correlation was found between the biological values calculated by the methods of *Oser* and *Speidel & Bennett* with a correlation coefficient (r) of 0.999 (Fig. 1). In view of the slight difference between the formulae of these two methods, such a close correlation was, of course, to be expected. The correlation between the *Mitchell & Block* and the *Oser* methods (Fig. 1), as well as that between the *Mitchell & Block* and *Speidel & Bennett* methods was similarly good, resulting in values of correlation coefficients of 0.802.

No correlation was found when the biological value obtained by the method of *Korpáczy* and co-workers was compared with the biological values obtained by the *Mitchell & Block*, the *Oser*, and the *Speidel & Bennett* methods (Fig. 1), resulting in correlation coefficients of 0.126, 0.033 and 0.108, resp. The main deficiency of this situation is the fact that the proteins tested showed *Korpáczy* values in a very narrow range (58–65) only.

Table 8

Correlation between the various methods used for the evaluation of biological value of some fungal proteins

	Oser	*Korpáczy et al.*	*Speidel & Bennett*	*Mørup & Olesen*
Mitchell & Block	0.802	0.126	0.802	*0.467*
Oser	–	0.033	0.999	0.198
Korpáczy et al.	0.033	–	0.108	*0.429*
Speidel & Bennett	0.999	0.108	–	*0.457*

No correlation could be detected between biological values of *Oser* and *Mørup & Olesen*, but there was a loose correlation between the latter and the other investigated methods (Fig. 1), with correlation coefficients of 0.467, 0.457 and 0.429, resp.

A direct comparison of the correlation between the various methods of calculating the biological value of the proteins in the mould strains tested is given in Table 8.

3. Conclusions

The nutritive value of fungi was investigated many years ago (SKINNER *et al.*, 1933). Several fungi are traditionally applied in the food industry (cheese and salami production), too. The biomass of a *Rhizopus* sp. was incorporated into human diets during the Second World War, with satisfactory results (BHATTACHARJE, 1970). *Rhizopus* strains were further investigated for this reason (SHUKLA & DUTTA, 1965).

In our work *Rhizopus*, *Mucor* and *Actinomucor* species were cultivated in submerged culture utilizing complex waste material (waste bread) as the carbon source to obtain fungal protein for human food.

The maxima of protein production differed according to the strains and the media used. In waste bread medium the best protein yields of the *Actinomucor*, *Mucor* and *Rhizopus* strains were measured in the 48, 54 and the 42 h cultures (ZETELAKI-HORVÁTH *et al.*, 1975; ZETELAKI-HORVÁTH *et al.*, 1976; ZETELAKI-HORVÁTH & VAS, 1976).

In contradiction to the results of SHUKLA and DUTTA (1965) (30% protein content in the 72-h mycelium), our strains had 35–40% protein in the mycelia, while at the cultivation time of the maximum protein yield (48 h) this was even higher (ZETELAKI-HORVÁTH & VAS, 1976). The protein yields of the fungal strains could further be increased with better agitation and aeration conditions (ZETELAKI-HORVÁTH *et al.*, 1977).

Considering the amino acid spectra, sulphur-containing amino acids were present in limited quantities in the mycelia of all the investigated strains. The highest methionine and cystine content (1.98, 1.40 and 1.33) was found in the protein of *Rhizopus* strains (Nos. 221, 209 and 216, resp.).

The majority of the strains possess high lysine and tryptophan contents, which are the limiting amino acids in cereals. Thus, these could serve as suitable supplements for the protein mixtures of various foods.

The determination of the biological value of proteins for humans is of great importance.

All methods have numerous difficulties and possibilities for error, even the *in vivo* utilization experiments with animals and humans.

KOFRÁNYI and co-workers (1970) carried out very accurate, long-term N-balance experiments with humans using foods of different composition. They found a mixture of potato and egg to have the highest biological value for humans (KOFRÁNYI, 1973), 35% higher than that of whole egg. The biological value determinations with animal feeding tests have the disadvantage of requiring a long period for the utilization test. On the basis of the results of KOFRÁNYI and co-workers (1970), MØRUP and OLESEN (1976) worked out an equation for the prediction of the biological value of proteins, while KERESE (1976) tried to simulate the digestion of humans by enzymatic *in*

vitro digestion experiments to ensure the highest resemblance to natural conditions.

In the first part of our selection work, preliminary information was necessary about the biological value of the investigated strains. This is why the amino acid content of the shaken cultures was determined and biological values were calculated according to the "chemical score" (MITCHELL & BLOCK, 1946), essential amino acid index *(EAA;* OSER, 1951), *EAA*, completed with a factor of digestibility (SPEIDEL & BENNETT, 1972), to total amino acids (KORPÁCZY *et al.*, 1961) and to the equation of MØRUP and OLESEN (1976) based on the *N*-balance experiments of KOFRÁNYI (1973).

It must be stated that the *in vivo* experiments cannot be replaced by chemical methods, but fast methods for the determination of biological value are indispensable in the course of laboratory experiments in any field of protein research. On the basis of the comparison described above, methods based on amino acids that cannot be synthetised by the human organism appear to be preferable.

*

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ENHANCED EFFICIENCY OF PRODUCT DEVELOPMENT AND ENGINEERING *VIA* CONSUMER FEEDBACK

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Product engineering has been coupled with refined product assessment of sensory and acceptance characteristics. The link becomes a model interrelating ingredients (or process variations) with sensory/acceptance responses. This model serves as a utilitarian purpose. First, the model allows the product developer to estimate the likely change in sensory/acceptance reactions to specified product changes. Second, the model allows the product developer to interpolate, and ascertain the optimally acceptable product, within ingredient constraints or within ingredient and cost constraints. Third, the model allows the product developer to estimate the likely ingredient combinations which generate a specified sensory profile.

These three benefits in concert provide for product engineering an integrated new look. Technical know-how, consumer polling, and business oriented optimization combine into a smoothly functioning system to enhance efficiency and profitability.

Consumer evaluation of product quality and acceptability has developed into an important aspect of the food industry. Acceptable products will be repurchased (assuming adequate marketing), whereas unacceptable products will be rejected, and the investment costs in manufacturing could well be lost.

How can the product developer, the engineer, as well as those involved in the marketing of food products assure themselves that their product has been optimized to accord with consumer tastes. Traditional early stage product development relies on expert 'flavor profile panels' as proxies for the consumer. These approaches are time-consuming, for one must carefully train a panel. At the same time one cannot be confident that these expert judgements truly represent current consumer needs and desires.

This paper concerns optimization technology in product development and consumer testing. Optimization provides the developer enhanced efficiency in formulation to keep pace with changing (or new) consumer needs/wants. Optimization also integrates cost of goods into a development/marketing strategy to provide another direct benefit.

1. The rationale of a product optimization analysis

In order to optimize the product developer should quantitatively interrelate ingredients, cost, consumer perceptions and acceptance. Interrelations, in the form of equations linking ingredient/processing variables to consumer acceptance/rejection and to cost-of-goods, allow the product developer or the process engineer an idea of how changes in product formulation will be perceived and accepted.

The product developer can construct models of consumer perceptions in a straightforward manner, provided that he or she has done the following preparatory steps:

- Narrowed the range of ingredients/process variations to a manageable set
- Developed a range of alternative test formulations, encompassing different levels of the ingredients, varied according to a systematic experimental design
- Selected the appropriate target group of consumers who evaluate the products
- Developed the test instrument (generally a questionnaire probing product perceptions/product acceptability)
- Integrated the resulting data into a model
- Utilize the model to explore alternative ingredient formulations in a search for optimal acceptability.

2. A specific example: sauce optimization

The goal was to develop a 3-component sauce which was highly acceptable, and at the same time cost effective. The 3 sauce components under direct experimental control were thickener, spice level and solids level. The 3 ingredients could be varied through an infinite set of combinations, each of which would generate a unique set of sensory impressions of taste, aroma and texture. Some of these variations would be highly acceptable, others would be modestly acceptable and still others would be unacceptable.

To enhance the efficiency of product development the 3 ingredients varied to generate an experimental design comprising 15 alternative formulations. These 15 formulations, represent a much larger array of alternative products that can be tested.

Statistical experimental designs provide for the engineer, food scientist and agricultural engineer, alternative design plans, whereby a small set of variations generate sufficient information to build the model. Table 1 shows the range of variation and the experimental design for the 15 sauce products.

Table 1

Experimental design of the study ("Central composite")

Product	Ingredient		
	Spice level	Solids	Thickener
1	H	H	H
2	H	H	L
3	H	L	H
4	H	L	L
5	L	H	H
6	L	H	L
7	L	L	H
8	L	L	L
9	H	M	M
10	L	M	M
11	M	H	M
12	M	L	M
13	M	M	H
14	M	M	L
15	M	M	M
Ingredient concentration (Relative units)			
H = High	1.75	1.75	1.75
M = Medium	1.0	1.0	1.0
L = Low	0.25	0.25	0.25

Although many thousands of alternative sauce variations could have been developed, these are unnecessary, and do not add additional information beyond the 15 tested here.

It is interesting to note that traditionally the R & D group would probably expend the same effort in developing test sauce products but would do so less systematically and over an extended period of time. The current approach telescopes product development 'fine tuning', from a small scale long term project, into a larger scale short term effort.

The reader should bear in mind that in some instances these sauces will be highly acceptable, whereas for other test variants the sauces will taste terrible and be rejected. The product developer must guard against the temptation of initially sorting through the wide variation in order to test only those products which he or she feels will pass muster. A crucial aspect of product optimization is to obtain data on each of the test formulations, acceptable or not, in order to ascertain ingredient levels where acceptability turns down from the optimum, and levels where acceptability turns to rejection.

3. The target group: consumer or experts?

The next step in product optimization is to select the appropriate group to these variations. Quite often product developers use in-house 'expert' or in-house 'consumer' panels. These individuals provide biased data, for several reasons:

- In-house expert or consumer panels may not reflect the consumer. Their tastes may not reflect the consumer tastes. Their sensory perceptions of products may have been unusually fine tuned as a consequence of continued exposure and retraining with the product category, time and time again.
- Consumers, however, do reflect the ultimate users. Consumers should be the panelists or testers, unless cost or other considerations preclude them. In such unusual instances the manufacturers should search for a surrogate group to replace consumers, and develop a transformation equation or rule which predicts consumer reactions from the reactions of the surrogates.
- The panelists should be selected from different regional and age groups (which are often unavailable in-house). Quite often "tastes" or acceptance ratings vary from one group to another. Unlike invariants such as engineering efficiency of a process, consumer reactions vary across locations, ages (children show different patterns of liking compared to adults) and brand usages (quite often users of one brand show entirely different ratings of product acceptability compared to users of another brand, even though both groups sensorically rate the products identically).

A valid test instrument for product acceptability is either a well developed questionnaire or behavioral measures, such as product volume purchased during an extended period. For most product optimization studies, long-term behavioral measures cost too much and are inefficient. By and large early product-development and optimization rely upon questionnaire data and evaluations after a short term product exposure wherein panelists evaluate the product and scale their reactions.

Product testing occurs in each of three different modes. Each mode necessitates a different type of questionnaire. The modes are:

Long-term home use test. The panelist receives one or two products (either through the mail or hand-delivered by an interviewer). The panelist consumes the product during an extended period (up to 4 weeks), and then scales his or her reactions. The home-use test permits panelists to experience the product in depth, under long-term use situations. Home use testing is expensive for product optimization, because it requires many more families to evaluate a

large number of products (*viz.* 15) than to evaluate just one or two products. Generally product developers do not use home use tests during the early phase of optimization.

Central location, prerecruit. Panelists test some or possibly all of the alternative product variations. They scale each product separately on the series of attributes. The testing may require several hours. The procedure maintains sensitivity, however, and is efficient. It allows 10 min rest between samples, to insure adequate sensitivity. We used this method for the sauce optimization. Each panelist scaled all 15 variations. (If 30 or so variations are of interest, each panelist might evaluate a unique 50% of the sample set.) The central location, prerecruit method provides the optimally efficient procedure for valid data collection.

The procedure is eminently utilitarian, permitting assessment of many different alternatives under well controlled test conditions. Thus, it generates the required data base very quickly.

Central location, intercept. An interviewer intercepts panelists at a mall, and tests them in a one-to-one situation. Like the home-use test, the panelist may test 1–4 different variations. A limitation of the intercept test for product optimization is the extensive interviewer time required. The interviewing is prone to biases generated by the idiosyncratic interaction of interviewer and panelist.

3.1. Questionnaire format

Questionnaires for product optimization probe perceptions of three distinct types of characteristics.

– *Sensory attributes.* Attributes such as flavor strength, redness/brownness, thickness/viscosity in the mouth represent straightforward sensory characteristics. They are unambiguous. They reflect the "more" physically correlated perception, and are often highly correlated with physical ingredients or processes. Furthermore, the more objective characteristics usually produce ratings with relatively low inter-panelist variability. Most panelists have a fairly good idea about and agree with each other regarding the sensory characteristic and its magnitude, when evaluating a specific product.

– *Performance characteristic* (liking, purchase intent, liking of specific characteristics). These attributes measure hedonics, or evaluative criteria. They are often not correlated in a simple 1 : 1 linear manner with physical stimulus levels. Quite often liking increases with increasing physical level, maximizes at an intermediate level and then diminishes as the physical level further increases.

– *Image characteristics* (*e.g.* appropriate for children, appropriate with various meats, an expensive sauce, *etc.*). Image attributes integrate the sensory

Table 2

Attributes tested in this study

Performance	Sensory	Image
Overall liking	Flavor strength	Richness of taste
Purchase intent*	Spiciness	Homemade taste*
Purchase intent ⓐ 89 ¢ /Jar*	Thickness	Appropriate for children's food*
	Slippery texture*	
	Aroma strength*	
	Depth of color*	

* Not discussed here.

information, along with consumer's perception of how the product fits into the meal, *etc.* Image characteristics may or may not be correlatable to physical ingredient levels. Nor in fact is it necessary that an image characteristic obey any lawful relation whatsoever, *vs.* physical ingredient levels.

The attributes used for the study are shown in Table 2. Note that each of the types of attributes appears. A profile of the product on these attributes becomes a snapshot as to how panelist perceives the product, from several viewpoints, including sensory, hedonic/performance, and image. The profile emerges as a valuable tool for consumer feedback, when changes in product formulations are required to enhance acceptability.

The questionnaire comprises three portions:
- Orientation
- Product/concept/ideal/profiling
- Calibration

3.2. Orientation

Traditional scaling uses a fixed point scale (0–9, 0–5) to reflect graded degrees or "amount". Other evaluation methods force panelists to select which of two samples is preferred, which is sweeter, which sweetness is more acceptable, *etc.* The former fixed point scale (*viz.*, 0–9) possesses severe technical limitations. The fixed point scale does not validly measure differences among products. What does the panelist do when rating a product for tartness or sourness, when the top point of the scale, "9" has been used? Suppose a tarter product is now evaluated. Why can not a 10 be used? Furthermore, with paired comparison testing (*viz.* which product is sweeter) there is no scale at all. The answer is *all-or-none.* Either product "A" is sweeter than "B" (but we don not know by how much) or "B" is sweeter than "A" (but again, we do not know by how much).

This study used a more scientific, rational scaling procedure, known as magnitude estimation (STEVENS, 1975). Magnitude estimation allows panelists to rate products using numbers of their own choice. The only limitation is that ratios of numbers should reflect ratios of perception. If product "A" is twice as sweet as product "B", then the panelist must assign to "A" a number twice as large as assigned to "B", and so forth.

Scientists have used magnitude estimation during the past 26 years to discover laws relating to ingredients or physical stimulus magnitudes to perceptions. Magnitude estimation generates validated ratio scale numbers (similar to the ratio scale numbers delivered by measures of weight, absolute temperature, *etc.*). It allows panelists to use their own frame of reference, and allows them to use a continuous scale. These properties ensure enhanced sensitivity, and an ability to accurately assess differences when these differences exist.

In order to orient panelists in magnitude estimation we used a straightforward procedure. Panelists scaled shapes of different areas (circles, squares, triangles), assigning numbers to these shapes to reflect perceived areas. Table 3 shows how these numbers track the physical area. (The relation between rated area and actual physical area is monotonic increasing, but conforms to a power law, rather than a linear law.) Panelists find orientation easy and enjoy it. Furthermore, panelists quickly see that a limited scale, *viz.*, between 0 and 5 or 0 and 9, does not reflect the range of physical areas.

In order to orient panelists to liking/disliking ratings (for evaluation of the performance characteristics) we used a slightly different procedure. Panelists first categorized words as to liked (by writing an „L") or disliked (by

Table 3

*Magnitude estimates assigned to shapes of varying area**

Circles		Triangles		Squares	
Area (cm^2)	Rating	Area (cm^2)	Rating	Area (cm^2)	Rating
7.1	7.8	2.0	4.7	10.1	13.2
19.7	10.0	7.4	10.7	17.9	13.8
43.0	31.1	24.8	22.5	72.4	47.6
91.6	52.2	64.9	49.7	123.0	68.5
145.3	69.5	104.0	65.4	123.0**	69.5
216.4	106.9	322.0	92.4	203.0	97.4
Power Law Exponent	.81		.62		.67

* 22 Panelists
** Reliability test

writing a "D"). Then they scaled degree of liking or disliking. Small numbers reflected low degrees of liking or disliking. High numbers reflected high degrees of liking or disliking. A 0 reflected neutrality. This exercise also was quite simple, and illustrated the principle of bipolar scaling. Furthermore, the exercise showed them a wide range of possible numbers to use for liking and disliking.

Table 4 shows the average ratings for the words.

Table 4

Magnitude estimates assigned to words

Sex	138
Love	126
Kiss	84
Money	82
Sun	73
Flavors	58
Puppy	47
Spaghetti	41
Perfume	37
New York City	0
Worm	–5
Mud	–25
Cigar	–31
Rattlesnake	–40
Pollution	–72
Hate	–81
Murder	–140

3.3. Probiling

After orientation, panelists received the first product (randomized, so that panelists began with different products). Panelists profiled the product on various attributes, assigning ratings in the same way that they assigned ratings to the words (for liking/disliking) and to the shape (for amount). The first product was an orientation product further illustrating the test. During their evaluation of this product panelists could further clarify the meaning of specific characteristics. (Quite often some characteristics may be ambiguous, or too technical. Panelists often have difficulty with those alternatives.)

Afterwards, the panelists evaluated the entire set of products, using the same scale, and attributes. This procedure generated a data base comprising

ratings on each product. Quite often the number of products may be quite large. No single panelist can evaluate all the products. In these instances, balanced incomplete block designs can assign subsets of the products to different panelists. These designs insure that each panelist rates different products, and that each product is rated by the same number of panelists.

Afterwards, panelists profiled their ideal product using the same attributes and scales that they had used to rate the actual products. Profiles of an "ideal" product allows the product developer to ascertain the perception of the current product, and to determine what panelists would like their ideal product to be like. The profiles of actual products provide a stable empirical reference point, against which to check the ideals.

3.4. Calibration

Panelists freely scaled their product perceptions, using any scale with which they felt comfortable. This freedom augments discrimination of differences among products, but it introduces substantial, extraneous and non-critical variation among the numbers used by different panelists. Thus, a normalization, or indexing procedure subsequently coalesced the scales used by different panelists. The key benefit in calibration or normalization is that with indexing one maintains the ratio properties of the ratings, while simultaneously eliminating a substantial portion of the extraneous interpanelist variability. The calibration procedure appears in Table 5. At the end of the product evaluation each panelist assigned numbers, using her personal scale, to reflect "extreme", "very much", "moderate", "slight", and "none at all = 0" (by convention, since this is a ratio scale).

Table 5

Example of "data" calibration for a panelist.
Typical magnitude estimation ratings for a panelist for sweetness, flavor intensity, spiciness

Before calibration

Package "017"

Calibration		Product rating	
Extremely	160	Sweetness	47
Very	128	Flavor intensity	83
Moderately	90	Spiciness	94
Slightly	35		
None	0		

After calibration

New M. E. = (Old M. E.)/(Pivot) × 100

Pivot = (Extremely + Very + Moderately + Slightly)/4

Calibration		Product rating	
Extremely	155	Sweetness	46
Very	124	Flavor intensity	80
Moderately	87	Spiciness	91
Slightly	34		
None	0		
Pivot =	103.25	Pivot =	43.25

4. Results

4.1. Data base development

The foregoing procedure efficiently develops a data base for consumer-based product optimization. Since there are different products, systematically

Table 6

Ingredient level			Sensory level					
Spice level	Total solids	Thick-ener	Overall liking	Rich-ness	Flavor strength	Thick-ness	Spici-ness	Cost
1.75	1.75	1.75	15	40	87	49	55	3.025
1.75	1.75	.25	29	20	80	27	50	2.687
1.75	.25	1.75	40	36	21	35	61	2.313
1.75	.25	.25	30	22	25	18	56	1.937
.25	1.75	1.75	20	35	95	43	39	1.563
.25	1.75	.25	18	24	74	25	34	1.187
.25	.25	1.75	29	44	15	64	26	.813
.25	.25	.25	14	16	32	12	30	.438
1.75	1.00	1.00	67	27	51	31	58	2.500
.25	1.00	1.00	30	22	40	22	30	1.000
1.00	1.75	1.00	54	30	88	38	43	2.125
1.00	.25	1.00	48	40	17	34	45	1.375
1.00	1.00	1.75	76	51	45	67	50	1.938
1.00	1.00	.25	54	18	39	14	39	1.563
1.00	1.00	1.00	49	27	47	30	43	1.758

varied on ingredients, and since one can estimate item cost, one can discern interrelations among ingredient levels, acceptance, product perceptions and product cost.

Table 6 shows the data base for five attributes of the 12 tested and total cost. The data base represents average ratings from 45 panelists. It could be augmented by:

- Segmenting the data by different subgroups in the population (age, usage pattern, income, *etc.*)
- Evaluation by panelists of the same products, but this time using many more attributes or characteristics, to further "flesh out" the profile (beyond the 12 tested and the five reported here).

4.2. Understanding product perceptions

The first stage in optimization with consumer feedback develops shorthand summaries of how sensory characteristics relate to ingredients. Traditionally a linear equation of the form

$$S = k_0 + k_1 A$$

relates perceived sensory intensity (S) to physical intensity (A), for a one ingredient system. For a two ingredient system, a plane of the form

$$S = k_0 + k_1 A + k_2 B$$

describes the data. Expressions 1 and 2 are parsimonious empirical equations, which may or may not provide the best fits to the data. (Other, non-linear, equations, comprising square terms and cross products terms are also feasible, but often are not necessary to accurately model sensory reactions.)

Table 7 shows the six linear equations, and both partial and multiple correlations. Some characteristics can be described by linear equations, whereas others (most noticeably "liking"/disliking) are not quite as well fit.

Table 7

	Regression equations				Correlation analyses			
	Intercept	Spice level	Total solids	Thickener	Multiple R	Spice level	Total solids	Thickener
Overall liking	28 20	10.67	–4.67	3.33	.38	.34	.15	.11
Taste richness	16.67	.53	–1.20	14.13	.86	.03	–.07	.86
Flavor strength	5.73	1.07	41.87	1.73	.96	.02	.96	.04
Perceived thickness	10.60	–.80	2.53	21.60	.84	–.03	.10	.84
Perceived spiciness	24.47	16.13	.40	2.93	.95	.93	.02	.17
Cost	.01	.99	.49	.25	1.00	.87	.43	.22

Linear equations indicate to the product developers or engineers the likely sensory change given specific changes in the ingredient or processing levels. If the partial or multiple correlations between the ingredients and the sensory ratings are high, then the ingredients impact on the sensory perceptions. Sometimes the correlations are low, or one partial correlation is close to 0. This suggests lack of predictability of a specific sensory characteristic, or possibly that changes in the ingredient may not produce sensory changes. It may also indicate a need for a more complicated equation.

4.3. Model liking

In 1932 BEEBE-CENTER discussed the issues involved in liking/disliking or acceptance/rejection. This issue appears to have been first formulated by Wundt in Leipzig in 1879. Wundt speculated that as physical intensity or sensory intensity increased, liking first increased as well. In the middle intensity range, liking maximized, reaching a "bliss point" of optimal acceptability. Further increases in physical level did not increase liking, but rather exerted no effect or else diminished liking. (The example of sugar in coffee reflects this well. Increasing the sugar level first increases liking, but after the optimal level is reached, liking maximizes. Finally, additional sugar only diminished acceptability as the coffee passes from optimally sweet to "too sweet" for the consumer's taste.)

In order to adequately model liking, which reaches a bliss point at an intermediate level, one should use the quadratic equation, comprising linear, square and cross product terms. The quadratic function better describes how liking varies with changes in the physical ingredients. The quadratic equation permits (but does not require) an intermediate bliss level (should one exist), and accounts for some ingredient interactions as well.

Table 8 compares the linear and quadratic function for liking. The linear equation is more parsimonious. On the other hand, the linear equation fails to describe the date adequately and thus provides a spurious function which does not permit estimation of the optimally acceptable formulation.

With non-linear optimization and the quadratic function the product developer can determine with fair precision the location of the bliss point. During the optimization procedure, one should:

- Check how well the model fits the data
- Make the model more parsimonious by dropping extraneous terms, which add little or no predictability. This will lessen the possibility that the model is fitting "noise".

Table 8 shows the optimal ingredient levels. Furthermore, given these ingredient values, and given linear equations (in Table 7) relating ingredients

to perceptions, one can *estimate* the likely sensory profile which would be assigned to the optimal product. This likely sensory profile is shown below the optimal "recipe".

Table 8

Comparison of linear and non-linear equations relating overall liking to ingredients

(A) Linear: Liking = 28.2 + 10.7 (Spice level) −4.7 (Solids) +3.3 (Thickener)
R = 0.38

(B) Non-linear: Liking = −31.9 + 85.8 (Spice level) +69.7 (Solids)
−27.9 (Thickener) −35.2 (Spice)^2 −2.4 (Spice) (Solids)
−2.4 (Spice) (Thickener) −30.7 (Solids)^2
−10.4 (Solids) (Thickener) −5.8 (Thickener)^2
R = 0.92

	Overall optimal	Cost ≤ 1.5	Cost ≤ 1.1	Cost ≤ 0.90
Spice	1.14	0.86	0.58	0.43
Solids	0.85	0.74	0.63	0.58
Thickener	1.39	1.10	0.82	0.68
Liking =	66.1	61.8	49.1	39.7
Taste richness	35.8	31.7	27.8	25.8
Flavor strength	45.0	39.6	34.3	31.6
Thickness	41.8	35.5	29.4	26.4
Spiciness	47.3	41.8	36.4	33.7
Cost	1.9	1.5	1.1	0.5

4.4. Constrained optimization

We have primarily dealt with single constraints on ingredients; *i.e.*, optimal ingredient levels should lie within the tested range of ingredient variations. During the optimization the ingredient levels could not exceed any of the limits tested in the study (although they may lie at the boundary). Beyond that region there is no data to estimate the shape of the liking surface *vs.* ingredients. Hence extrapolation is unwarranted.

Let us now introduce another constraint. *viz*, a linear combination of ingredients lies within specified boundaries. This additional *linear constraint*, over and above the ingredient or point constraints, further reduces the feasible region of optimal products. The linear constraint could be a cost-of-goods constraint. Since each ingredient (or process level) entails a specific cost, one should be able to optimize overall liking, while at the same time

- maintain the formulation within the range of previously tested ingredient levels
- maintain total cost within prespecified levels.

Table 8 shows three of these alternative recipes, subject to different cost constraints. Were cost not constrained then the optimal product would still entail a finite cost (1.912 units). On the other hand, with successively reduced costs of goods as constraints (below 1.912) the model suggests different alternatives. Acceptability, based on consumer ratings, diminishes. The optimization approach keeps acceptability as high as possible, given the ingredient and cost constraints. Furthermore, the product developer should be able to estimate the likely sensory profiles produced by the different recipes which satisfy the cost reductions. This added benefit ascertains whether for a specific cost reduction liking diminishes too much, or some undesirable sensory characteristics emerge, owing to the lowered ingredient levels, and the change of ingredient balance.

5. Profile matching optimization

A second issue in product formulation/optimization is to match an existing profile, using one's own ingredients. Quite often an existing product is acceptable, but economic conditions or distribution factors have made the current cost-of-goods too high, or ingredient availability too low, so that the recipe is not economically viable. The product developer must imitate the sensory characteristics of the current product, using less expensive, or perhaps more available ingredients.

The traditional, time-honored procedure requires that product developer formulate a variety of alternative formulations, using the new ingredients. When the product seems to match the desired profile, the test formulation is submitted to an in-house or consumer panel for evaluation. In preparation for this test the product developer often creates numerous alternative recipes, but not systematically to create a necessary data base.

The data base developed in Table 6 interrelates ingredient levels and perceptions. Given ingredient levels for the 3 constituents (which have already been selected, and agreed upon) the product developer can predict the likely sensory profile, using the linear regression equations shown in Table 7. Since these regression equations summarize the interrelation between ingredients and perceptions, they model the product.

Let us turn the issue around. We have a product profile which we would like to produce. The queries are:

- With the current ingredients in the data base (Table 6) and within the limitations of the ingredients, can we match the prespecified target profile?
- What is the ingredient formulation for the match?

With the technique of goal or multi-objective programming (MOSKOWITZ *et al.*, 1977; who called it the "Eclipse" system) one can estimate the likely ingredient combination which comes as close as possible to producing the desired profile. Of course this estimate is just that – a best guess, based upon consumer perception of the products in the data base. The manufacture/product developer should validate this best estimate through subsequent testing. The net benefit, however, is that the effort is efficiently channeled in an optimum direction towards reducing product development time. The same effort would have been expended in making the alternative formulations, but not systematically. What is new, however, is the development of an integrated data base, *via* disciplined product development.

Table 9 shows these results for 3 prospective profiles, using the data base, and the equations in Table 7. The aim in Table 9 was to determine what combination of the three ingredients would produce a matching profile given each different profile as a target.

Table 9

Examples of profile matching to prespecified profile

	Profiles		Ingredients generating profile		
	Desired profile	Obtainable profile	Spice level	Total solids	Thickener level
(A) Taste richness	50.0	39.0	0.30	1.46	1.75
Flavor strength	70.0	70.0			
Perceived thickness	70.0	51.8			
Perceived spiciness	35.0	35.0			
(B) Taste richness	35.0	35.0	0.68	1.34	1.39
Flavor strength	65.0	65.0			
Perceived thickness	55.0	43.4			
Perceived spiciness	40.0	40.0			
(C) Taste richness	40.0	34.5	0.71	0.75	1.30
Flavor strength	40.0	40.0			
Perceived thickness	40.0	40.0			
Perceived spiciness	40.0	40.0			

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EVALUATION OF POSSIBLE MUTAGENICITY OF IRRADIATED SPICES

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After establishing technological and economic feasibility of radiation decontamination of spices, extensive studies have been initiated to test the wholesomeness of irradiated spices.

As a part of this program of wholesomeness testing, investigations were carried out on the possible mutagenicity of ground paprika, black pepper and a spice mixture untreated or radiation treated at 5, 15 and 45 kGy dose levels, respectively. The composition of the spice mixture was as follows: paprika, 55%, black pepper, 14%, allspice, 9%, coriander, 9%, marjoram, 7%, cumin, 4%, and nutmeg, 2%. Studies were performed using the *Salmonella/microsome* test of various extracts of spices and an *in vivo* assay of urine metabolites from rats fed with a diet containing spices. Urine was collected after 6 days feeding with spice-containing diets. Indicator organisms were histidine auxotrophic *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100. Investigations were performed within 14 days subsequent to the radiation treatment of spices and after 90 days storage of the irradiated spices, resp. Known mutagenic substances (aflatoxin B_1, streptozotocin, α-naphthylamine and sodium azide) served as positive controls in the mutagenicity tests.

Neither samples of the spice extracts nor urine samples induced a significant increase in the frequency of revertants in the *Salmonella* test system.

Ionizing radiation at a dose level of 5 kGy is effective in reducing the viable cell count of microorganisms in spices to such an extent that they no longer contribute significantly to the microbial contamination of the food into which they are incorporated (FARKAS, 1973; FARKAS *et al.*, 1973; KISS *et al.*, 1978). After the establishment of the technological and economic feasibility of radiation decontamination of spices, extensive studies have been initiated in Hungary to test the wholesomeness of irradiated spices as a contribution in kind to the International Project in the Field of Food Irradiation (IFIP, Karlsruhe, FRG). Preliminary rat feeding studies already performed with irradiated spices did not indicate any toxic or teratogenic effect (BARNA, 1973, 1976; JOINT FAO/IAEA DIVISION OF ATOMIC ENERGY IN FOOD AND AGRICULTURE, 1975; ANON., 1978).

As part of this program of wholesomeness testing, microbiological methods were applied to test potential carcinogens in untreated or radiation-treated ground paprika (capsaicin-free), black pepper and a spice mixture, respectively. Earlier mutagenicity testing of irradiated ground paprika has been already reported (IFIP, 1977).

1. Materials and methods

The composition of the spice mixture was as follows:

paprika, 55%,
black pepper, 14%,
allspice, 9%,
coriander, 9%,
marjoram, 7%,
cumin, 4%,
nutmeg, 2%.

Radiation treatment of spices was performed under aerobic conditions with a ^{60}Co gamma radiation source at ambient temperature.

In a preliminary study, extracts of the spice mixture have been tested for mutagenicity using the plate-incorporation procedure described by AMES and his co-workers (1975), who also generously provided us with the different test-organisms. The latter were histidine auxotrophic *Salmonella typhimurium* strains. One-gram samples of spice mixture radiation treated with 15 kGy and 45 kGy, resp., were extracted three times with 9 cm^3 acetone or ether. The extracts were evaporated to dryness in a rotating vacuum evaporator and the residues were taken up in 1 cm^3 dimethylsulphoxide. Aliquots (0.2 cm^3) of these solutions were tested for mutagenic activity with *Salmonella/microsome* test by incubation for 48 h at 310 K (37 °C) with the addition of a 9 000-*g* liver supernatant (S-9 fraction) isolated from Aroclor 1254-treated male Sprague-Dawley rats (AMES *et al.*, 1973). One hundred μg α-naphthylamine and 0.1 μg aflatoxin B_1 per plate, resp., served as positive controls.

In further studies, urine samples of albino rats fed a diet containing 25% spice mixture, 25% paprika and 3.5% black pepper, respectively, were tested with five *Salmonella* tester strains in the standard *Ames* assay. Untreated as well as 5 kGy irradiated samples of spices, along with the unirradiated controls, were tested in these experiments. Urine was collected after 6 days of feeding spice-containing diets, and was kept freeze-dried in a refrigerator until mutagenicity testing. For testing, freeze-dried urine samples were taken up in half of their original volume of distilled water. Two-tenths cm^3 aliquots of these double-strength urine samples were used per plate. This type of mutagenicity testing of the urine metabolites of spices was performed within 14 days subsequent to the radiation treatment of spices and testing was repeated after 90 days of storage of the irradiated spices at ambient temperature. Known mutagenic substances served again as positive controls.

Only those experiments were considered to be satisfactory in which the number of spontaneous revertants did not deviate by more than a factor of 2 from those reported by AMES for the particular strain (MATTERN & GREIM,

1978). Following the standard procedure we considered the response to be negative if the number of induced revertants compared to the spontaneous value was less than 2-fold (AMES *et al.*, 1975; MATTERN & GREIM, 1978).

2. Results

The results of these preliminary tests are shown in Fig. 1. As it can be seen at the applied concentration level neither the acetone extract, nor the ether extract of irradiated spice mixture induced a significant increase in the number of revertants in the *Salmonella* test system capable of detecting various kinds of frameshift mutagens.

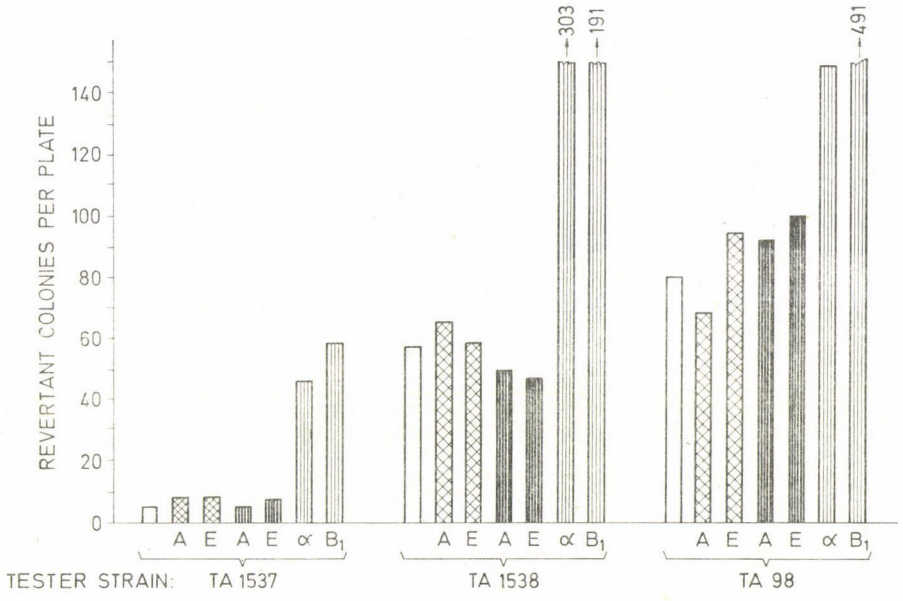

Fig. 1. *Salmonella/microsome* tests of extracts of the spice mixture. The columns in the Figure are average values of quadruplicates.
Legend: □ spontaneous; ▩ 15 kGy; ■ 45 kGy; ▯ positive controls; A = acetone-extract; E = ether extract; ∝ = 100 μg ∝-naphthylamine; B_1 = 0.1 μg aflatoxin B_1

Figure 2 shows the results of these mutagenicity tests with untreated and irradiated samples of the spice mixture fed to the rats for 6 days starting on the 7th day subsequent to irradiation. This Figure shows that neither the urine metabolites of untreated spices nor those of irradiated ones influenced the number of revertant colonies as compared to the spontaneous mutation

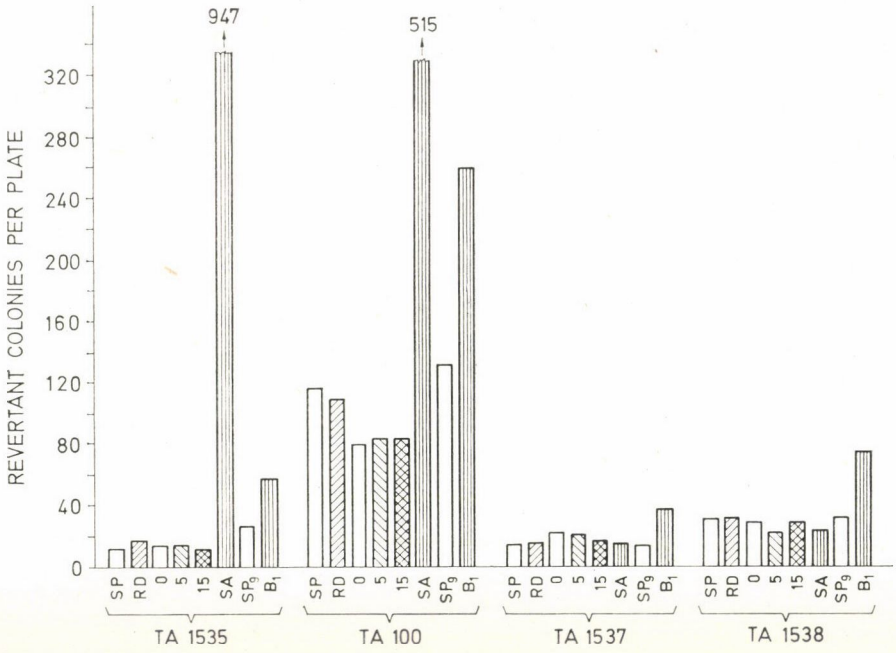

Fig. 2. Results of mutagenicity tests, without S-9 mix, of urine samples of albino rats fed a diet containing untreated and irradiated spice mixture, respectively. The mutagenicity tests were performed within 14 days after radiation treatment. The columns are average values of quadruplicates.
Legend: SP = spontaneous; RD = standard rat-diet; 0 = spice mixture, 0 kGy; 5 = spice mixture, 5 kGy; 15 = spice mixture, 15 kGy; SA = NaN_3, 1 μg per plate; SP_9 = spontaneous, with S-9 mix

frequency of the tester strains. From the positive controls, a highly significant increase in the revertant frequency was caused by sodium azide at a concentration of 1 μg per plate without microsomal activation among the cells of tester strains detecting base-pair substitutions (TA 1535 and TA 100). This compound was ineffective in the given concentration on the tester strains detecting frameshift mutagens. The revertant frequencies shown in Fig. 3 for sodium azide with strain TA 1535 and TA 100 are in good agreement with the literature (VAN KOOIJ *et al.*, 1978). All tester strains responded with increased number of revertant colonies to the 0.1 μg per plate level of aflatoxin B_1 when 0.5 cm^3 S-9 mix (AMES *et al.*, 1975) was incorporated into the system.

Figure 3 illustrates the results obtained with the urine samples from the rats fed paprika either in the unirradiated or in the radiation-treated state and stored for 90 days after irradiation. Here again, negative (no) response was observed with all tester strains.

The results obtained with urine samples from rats fed black pepper stored for 90 days after irradiation are shown in Fig. 4. In this case S-9 mix-

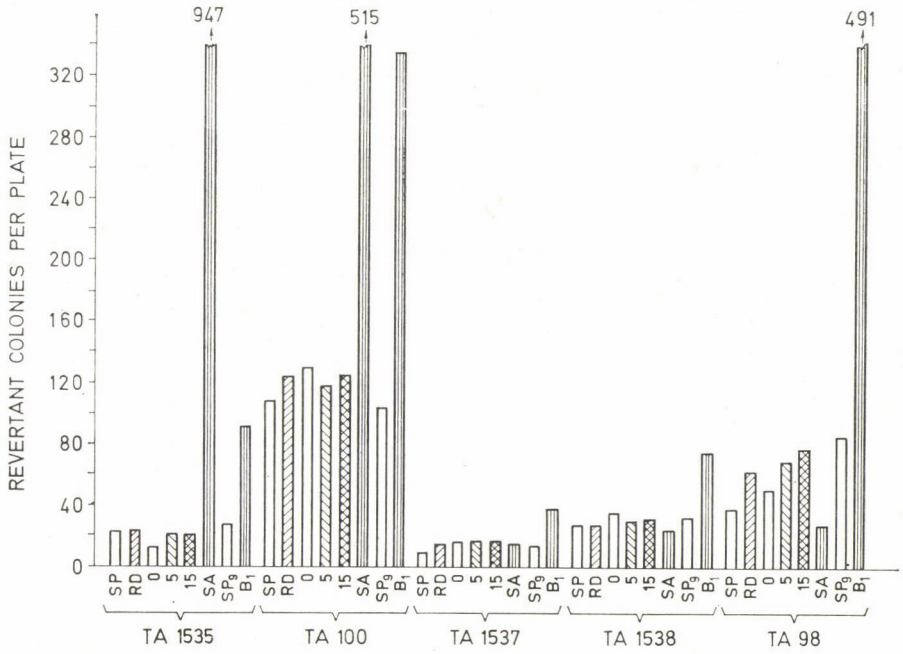

Fig. 3. Results of mutagenicity tests, without S-9 mix, of urine samples of albino rats fed a diet containing untreated and irradiated paprika, respectively. The mutagenicity tests were performed after 90 days storage of paprika subsequent to the radiation treatment. The columns are average values of quadruplicates.
Legend: SP = spontaneous; RD = standard rat-diet; 0 = paprika, 0 kGy; 5 = paprika, 5 kGy; 15 = paprika, 15 kGy; SA = NaN_3, 1 μg per plate; SP_9 = spontaneous with S-9 mix; B_1 = aflatoxin B_1, 0.1 μg per plate

ture was also incorporated to the plates testing urine samples. Streptozotocin at a concentration of 1 μg per plate was used without S-9 mix instead of sodium azide in this experiment. As with sodium azide, only tester strains TA 1535 and TA 100 responded to streptozotocin.

3. Conclusions

One can conclude from our observations shown only in part in these Figures that neither the extracts nor the urine metabolites of the spices investigated induced a significant increase in the frequency of revertants in the *Salmonella* test system. The samples studied in the preliminary tests with extracts were equivalent to 200 mg spice per plate. Considering the daily feed consumption of rats and the volume of urine produced by them on a daily average, the urine samples incorporated per plate were equivalent to the urine metabolites of 250–500 mg spice mixture or paprika and 35–70 mg

black pepper, respectively, consumed by a rat. A considerable further increase in the amount of urine seemed to us to be non-practical, taking into account its histidine content. The amount of urine used per plate in our experiments was based on the amino acid analysis of several urine samples. According to

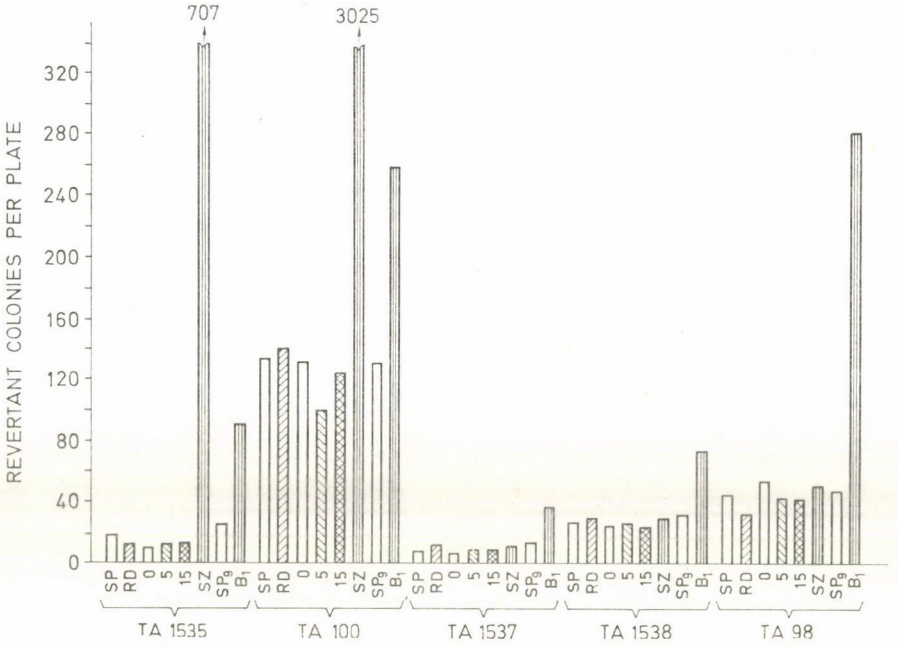

Fig. 4. Results of *Salmonella/microsome* tests of urine samples of albino rats fed a diet containing untreated and irradiated black pepper, respectively. The mutagenicity tests were performed after 90 days storage of black pepper subsequent to the radiation treatment. The columns are average values of quadruplicates.
SP = spontaneous; RD = standard rat-diet; 0 = black pepper 0 kGy; 5 = black pepper, 5 kGy; 15 = black pepper, 15 kGy; SZ = streptozotocin, 1 μg per plate; SP_9 = spontaneous, with S-9 mix; B_1 = aflatoxin B_1, 0.1 μg per plate

this investigation the histidine level in our urine tests was not more than 70 μg per plate. This histidine level was considered to be the maximum permissible without inducing heavy background lawn due to the excessive growth of all auxotrophic bacteria which would obscure the revertants (AMES *et al.*, 1975; VAN KOOIJ *et al.*, 1978). The standard Ames procedure incorporates 16 μg histidine per plate only.

Our Figures clearly show that, if mutagenic substances detectable by the *Salmonella* test would be present in the DMSO solution at a level of ca. 5 μg cm^{-3} (equivalent to ca. 5 μg per one g of spice mixture), or in the urine samples at a level of 2.5 μg cm^{-3}, they could probably be detected in our experiments.

The lack of mutagenicity of untreated and irradiated paprika, resp., was shown also in a host-mediated assay by earlier Hungarian studies (IFIP, 1977). Further tests for potential carcinogens using blood samples of the same rats, whose urine portions were already tested in the present studies, were performed (FARKAS & ANDRÁSSY, 1981) by the lysogenic induction of prophage lambda in *E. coli* according to the method of MOREAU and his co-workers (1976).

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PROPHAGE λ INDUCTION (INDUCTEST) OF BLOOD OF RATS FED IRRADIATED SPICES

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Lysogenic *Escherichia coli* K12 strains Nos. GY 5023: envA uvr$^+$ (λ) and GY 5027: envA uvrB (λ) were used as test organisms and *E. coli* strain No. GY 4015 as the indicator to investigate prophage induction *(Inductest)* of blood samples of CFY rats fed black pepper and spice mixture treated with gamma radiation. The dose levels applied for the irradiation of spices were 0.5 and 15 kGy. In the rat feed, the applied concentration of ground black pepper was 3.5%, and that of the spice mixture (: mild paprika, black pepper, allspice, coriander, marjoram, cumin and nutmeg) was 25%. Blood samples were taken for prophage induction after six days' feeding with the tested diet. Tests with pepper were performed both within two weeks after irradiation and again after 90 days of storage following irradiation, while with the spice mixture, *Inductest* was performed with the blood of rats fed a spice mixture irradiated 90 days before the start of the feeding test.

Neither the blood of rats fed irradiated pepper nor that of rats fed irradiated spice mixture did increase, to a statistically significant degree, the occurrence of prophage induction as compared with blood samples of rats fed a diet containing untreated spices or with commercial rat feed. In agreement with earlier microbial mutagenicity tests performed with extracts of irradiated spices and urine of rats fed irradiated spices proper, neither did present results indicating that spices irradiated with 5 and 15 kGy or their metabolites would be of DNA-modifying potential.

To detect whether gamma radiation causes development of agents with DNA-modifying potential in spices, spice extracts and urine of rats fed spices were tested in the assay system developed by AMES and associates (AMES *et al.*, 1975; FARKAS *et al.*, 1981). We thought it desirable also to test the blood of rats fed spices for the presence of genetically active agents. However, due to the histidine content of blood, it was not possible to perform a direct test of blood samples with the histidine-auxotroph test organisms used in the Ames assay procedure. For direct blood testing a prophage induction method (*Inductest*; MOREAU *et al.*, 1976; MOREAU & DEVORET, 1976) was tried which suggested by its elaborators as a similarly sensitive test to detect potentially carcinogenic agents. What is more, this test, in contrast with the *Salmonella* mutagenicity procedure (AMES *et al.*, 1975) is not sensitive to the possible microbial contamination of the samples, and we assumed that due to its nature, this method would not be disturbed by the nutritive value of the samples either.

1. Materials and methods

1.1. Irradiation of spices and feeding of rats

In the present investigations blood of those same rats was examined who's urine had already been tested by the *Salmonella/microsome* mutagenicity tests (FARKAS *et al.*, 1981). Conditions of spice irradiation and of rat feeding were also described in the above paper.

In the experimental feeding period the feed contained 3.5% of black pepper and 25% of spice mixture (composition given in the previous report of FARKAS and co-workers, 1981). The dose levels applied were: 0, 5 and 15 kGy. Blood of rats fed on commercial rat feed served as the comparison.

At each dose level, 5 non-inbred, 3 months old male CFY rats were fed through 6 days, and then 2 times 1 cm^3 blood samples were aseptically taken and 10 μl heparine added to each sample.

Samples were immediately quick-frozen or freeze-dried and were kept in this state in cold storage till later testing.

Black pepper was fed, and prophage induction of blood of such rats was performed within 2 weeks after irradiation and then again after 3 months of storage following irradiation. Spice mixture was similarly tested 90 days after irradiation.

1.2. The prophage induction procedure

Freeze-dried blood samples were suspended in sterile distilled water to original volume immediately before use. Tests were performed with two parallels of 0.4 cm^3 reconstituted blood samples per rat.

In the *Inductest*, lysogenic strains of *Escherichia coli* K12 Nos. GY 5023: envA uvr$^+$ (λ) and GY 5027: envA uvrB (λ) were used, the indicator bacterium was *E. coli* K12 strain No. GY 4015.

The test method was identical with that described as Inductest III in the publication of MOREAU and co-workers (1976). DMSO solutions of aflatoxin-B_1 and streptozotocin (1 μg per plate) were used as positive controls.

For testing positive controls, 0.2 cm^3 suspension of the lysogenic bacterium was mixed, instead of blood sample, with 10 μl of dimethyl-sulphoxide solution of carcinogenic agents. To define spontaneous induction, merely 10 μl dimethyl-sulphonate was added as a blind test to the above test system.

The viable count of lysogenic bacteria was determined by the dilution and the spread plate method, using DIFCO "nutrient agar" plates.

Liver homogenate fraction "S-9" was prepared from rat liver, induced with Arochlor 1254, by the method described by AMES and co-workers (1975).

2. Results and conclusions

The results are contained in Tables 1–6. After square root transformation stabilizing variance, infective centres per plate were compared by analysis of variance. No statistically significant ($P > 5\%$) difference could be detected in prophage induction by rat blood whether the animals were fed irradiated or non-irradiated pepper. The same was true for the spice mixture. Even at a concentration of 1 μg per plate (in the course of induction: 5 $\mu g\ cm^{-3}$) aflatoxin B_1 and streptozotocin had significantly increased the number of infective centres as compared with spontaneous induction frequency or with induction frequencies experienced in testing rat blood samples. Interestingly, in the case of one of the test organisms (Strain No. GY 5023), when using blood samples, induction frequency was smaller than the spontaneous induction frequency detected on plates containing the solvent of compounds used in the positive control.

Summarizing the results: in conformity with the results of our *Salmonella/microsome* mutagenicity tests (FARKAS *et al.*, 1981) performed with urine of rats fed irradiated spices and extracts of irradiated spices, prophage induction testing of the blood of rats fed irradiated spices did not indicate that spices treated with gamma radiation doses or their metabolites would *in vivo* possess a DNA-modifying potential.

Table 1

Prophage induction using blood samples of rats fed on feed containing 3.5% pepper

Irradiated spice was used for feeding *within 2 weeks* following irradiation.
Test organism: *E. coli K12 GY 5023*
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 1.53×10^3

Number of infective centres per plate										
Control samples			Blood samples*							
Spontaneous	Aflatoxin B_1 (1 μg per plate)	Streptozotocin (1 μg per plate)	Commercial feed		Feed containing pepper					
					0 kGy		5 kGy		15 kGy	
30	292	166	12	7	11	13	16	17	13	11
21	280	197	9	9	9	8	7	6	6	11
			11	12	3	18	13	14	5	13
			—	12	11	12	8	6	11	6
			12	8	5	17	9	15	13	12

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed
– Sample deteriorated

Table 2

Prophage induction using blood samples of rats fed on feed containing 3.5% pepper

Irradiated spice was used for feeding *within 2 weeks* following irradiation.
Test organism: *E. coli* K12 GY 5027
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 1.72×10^3

Number of infective centres per plate											
Control samples			Blood samples*								
			Commercial feed		Feed containing pepper						
					0 kGy		5 kGy		15 kGy		
Spontaneous	Aflatoxin B_1 (1 μg per plate)	Streptozotocin (1 μl per plate)									
45	752	62	52	53	56	43	56	58	52	46
47	548	67	55	35	49	59	56	39	49	60
			43	38	40	52	40	46	53	54
			45	34	46	38	29	39	45	51
			54	51	60	52	48	56	53	53

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed

Table 3

Prophage induction using blood samples of rats fed on a feed containing 3.5% pepper

Rat feeding with feed containing irradiated spice started *90 days after irradiation.*
Test organism: *E. coli* K12 GY 5023
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 1.01×10^3

Number of infective centres per plate										
Control samples		Blood samples*								
		Commercial feed		Feed containing pepper						
				0 kGy		5 kGy		15 kGy		
Spontaneous	Aflatoxin B_1 (1 μg per plate)									
11	218	5	7	7	7	8	8	1	10
13	257	5	8	10	10	8	5	8	7
		12	9	13	10	10	4	8	8
		6	9	4	7	7	8	9	6
		9	8	12	9	5	6	5	6

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed

Table 4

Prophage induction using blood samples of rats fed on feed containing 3.5% pepper

Rat feeding with feed containing irradiated spice started *90 days after irradiation.*
Test organism: *E. coli* K12 GY 5027
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 1.39×10^3

Number of infective centres per plate									
Control samples		Blood samples*							
Spontaneous	Aflatoxin B_1 (1 μg per plate)	Commercial feed		Feed containing pepper					
				0 kGy		5 kGy		15 kGy	
30	834	22	30	19	28	27	27	—	—
27	552	23	25	25	18	25	26	24	15
		32	19	25	22	26	17	35	20
		20	25	13	14	23	34	22	21
		21	27	18	17	20	17	21	20

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed
– Sample deteriorated

Table 5

Prophage induction using blood samples of rats fed on feed containing 25% spice mixture

Rat feeding with food containing irradiated spice mixture started *90 days after irradiation.*
Test organism: *E. coli* K12 GY 5023
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 0.97×10^3

Number of infective centres per plate									
Control samples		Blood samples*							
Spontaneous	Aflatoxin B_1 (1 μg per plate)	Commercial feed		Feed containing spice mixture					
				0 kGy		5 kGy		15 kGy	
27	916	20	26	30	23	30	24	23	24
31	1040	20	22	26	28	26	24	30	26
		32	15	19	36	29	18	30	26
		19	20	25	35	24	22	19	24
		29	30	24	29	34	30	28	31

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed

Table 6

Prophage induction using blood samples of rats fed on food containing 25% spice mixture

Rat feeding with feed containing irradiated spice mixture started *90 days after irradiation*
Test organism: *E. coli* K12 GY 5027
Number of lysogenic bacteria per plate: value estimated from the optical density measurement of stock suspension: 10^3
spread plate count: 1.10×10^3

Number of infective centres per plate									
Control samples		Blood samples*							
				Feed containing spice mixture					
Spontaneous	Aflatoxin B_1 (1 μg per plate)	Commercial feed		0 kGy		5 kGy		15 kGy	
23	105	3	6	8	10	4	4	8	5
23	137	4	1	3	11	5	4	7	2
		6	4	4	10	8	7	7	6
		7	3	2	10	12	12	8	11
		8	7	9	7	8	7	4	8

* Numbers next to each other in the same column mean two parallel blood samples from the same rat. Pairs of numbers below each other indicate 5 rats fed the same feed

*

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Abstracts

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ENZYMES AND OTHER BIOLOGICALLY ACTIVE AGENTS IN FOODS

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Of the biologically important groups of substances (vitamins, hormones, trace elements, enzymes) in foods the author discusses the enzymes.

Enzymes may be endogenous or deliberately added components of foods. They may also be used to detect and determine certain components of food materials of intricate composition.

Some endogenous (tissue) enzymes of foods and their raw materials are important in view of variety selection. Their activity values are characteristic of certain phases in the vital processes of the raw materials (growth, ripening, senescence), of some physiological changes, of infection and of stress effects. They are suitable for the control of storage or of technical operations (freezing, heat treatment, irradiation) or to show whether the latter had been carried out. Earlier knowledge in this field may be enhanced by the study of the different molecular forms of tissue enzymes.

Enzymes used as additives may be of animal, plant or microbial origin and may be added for different purposes, such as to increase yield, or to improve the efficacy of a technological procedure, to extend storage life or retard spoilage, to improve the quality of a product or to increase the economics of its production, *e.g.* by using a new raw material. Enzyme preparations used in the food industries have been lately produced in immobilized form. Their advantage lies in the fact that they may be used several times, they may be easily removed from the product, they are applicable in continuous processes and their stability is frequently higher. Their use, however, requires special techniques and equipment.

Another novel trend in the use of enzymes is the preparation of heat resistant preparations. Thereby, it is possible to accelerate technological processes by using higher temperatures. In the last decade, in spite of a certain slowing down, the use of new enzyme preparations has spread (*e.g.* glucose isomerase, endo-polygalacturonase, lactase) and earlier preparations entered new fields of food technology (*e.g.* the manufacture of protein concentrates).

The use of enzymes in food analysis is at present hindered by the high price of pure preparations. The use of immobilized products may advance this problem.

THE ROLE OF TRACE ELEMENTS IN FOODS

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The relationship between the inorganic elements present in our environment (geochemical sources, food) and human health was unambiguous even in the past. Man of historical times was only a passive final station of the effects, while to-day our food base forms an area of intense study and we exercise selection between various substances serving as foods.

Public attention started to focus on micro-elements only in the last decade. It has been known for about the last three thousand years which of the elements are toxic and to what extent (arsenic, lead, mercury). But the answers to the questions as to how much of the essential elements gets into our body and whether this amount may be considered optimal in the given situation are not exactly known.

These questions become more and more important because the micro-elements used increasingly in plant cultivation and in animal feed are changing the traditional proportions from biochemical point of view in the human diet. Due to the wide-spread application of chemicals, some important elements get into relative minimum and thus affect human health.

Based on earlier literature, the author is of the opinion that some diseases spreading rapidly in the last few decades, called also diseases of civilization, are, partially at least, the consequence of altered proportions of the nutrient elements.

CHANGES IN THE PEROXIDASE ACTIVITY IN APPLES DURING STORAGE

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The peroxidase enzyme takes part in the biosynthesis of ethylene and lignin. It is assumed, therefore, that, in case of fruits of climacteric ripening, it may be used as a marker of ripening and of the beginning of senescence. On studying the peroxidase activity in the apple variety *Golden Delicious* during cold storage in controlled athmosphere, two peaks of activity were observed. The first and higher one corresponded to ripening, while the other to the beginning of senescence (GORIN & HEIDEMA, 1976).

In order to find biochemical characteristics for the ripening of apples a comparative study was carried out. The specific peroxidase activity of *Golden Delicious* apples, other apple varieties and of apples stored in non-controlled atmosphere was followed during ripening and storage.

From 1976, the peroxidase activity was measured throughout 3 years in three apple varieties: *Jonathan*, *Starking*, *Golden Delicious*, picked at three different times. The activity was measured directly after picking and at the end of cold storage. Activity measurements were carried out in the apples from the second picking after storage in controlled atmosphere as well. The following observations were made:

– No correlation was found between the time of picking and the peroxidase activity. This was probably due to the fact that the apples picked in three subsequent years were not of the same stage of ripeness.

– At the end of cold storage the peroxidase activity in the apples was significantly higher than at the beginning.

– Activity increased also during storage under controlled conditions; the values fluctuated around those measured after cold storage.

– Both the levels of activity and the extent of increase depended on the year. The lowest value for all apple varieties was found in 1977, while the highest in 1978.

– The value of peroxidase activity depended to a certain extent on the variety. In a given year it was higher in the variety *Starking* than in the other two varieties.

To decide whether the determination of peroxidase activity is suitable to mark ripening and senescence, further and more detailed investigations are necessary. It may be established, however, that the variety *Golden Delicious* does not take up a special place in this respect.

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CHANGES IN CYTOKININ ACTIVITY OF STRAWBERRIES AS A FUNCTION OF RIPENING AND STORAGE

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The cytokinin activity of strawberry varieties *Gorella*, *Rival* and *Pokahontas* was investigated during ripening and storage.

Tests were carried out at the green, pink and red stages of ripeness. Samples of every variety were taken at the same time.

Storage was carried out at 298 K (25 °C) temperature and 60—70% relative humidity.

The cytokinin activity was determined by the *Amaranthus* test. The results permit the establishment of the following:

– the cytokinin activity of the strawberry varieties investigated decreased with ripening;

– of the varieties tested, *Pokahontas* showed the highest endogenous cytokinin activity; the second was *Rival;*

– during storage, the cytokinin activity decreased more slowly in variety *Rival* than in *Gorella*. No storage test was carried out with variety *Pokahontas;*

– storage technological investigations proved variety *Rival* to be better than variety *Gorella*.

AMYLASES IN VEGETABLES

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The amylase activity of various cauliflower and onion varieties was studied.

The vegetable sample was homogenized with a 5% solution of common salt and the filtrate was centrifuged. The amylase actvity in the supernatant was determined by the *Phadebas* method photometrically.

In cauliflower, amylase activity upon picking was 500–1000 U kg^{-1}. During 15 days of storage at room temperature, the activity increased to 1500–3500 U kg^{-1}. First, the increase of activity was of an accelerating, later of a saturation character. The activity in early varieties *(Avans, Clon)* was about 2–2.5 times lower than that of the late varieties *(Igloo, Elgon, Mátra)*. During storage at 4 °C, the enzyme activity amounted to only about 50% of that measured at room temperature and showed an almost linear increase as a function of time. Under the conditions of the study, the early varieties were found better than the late varieties (better keeping quality, lower alfa-amylase activity).

The onion samples (varieties: *Alsógöd, Aroma, Bolero, Favorit, Rivato, Tétényi Rubin)* showed a practically uniform activity (100–250 U kg^{-1}) during the first 5 months of storage. Parallel to shooting, the alfa-amylase activity increased to 300–900 U kg^{-1}. In some of the varieties *(Alsógödi, Aroma)* it reached a maximum.

The cauliflower and the onion varieties differed substantially. The results permit the possibility of drawing conclusions from the amylase activity as to the expected storage life of certain vegetables.

CHANGES IN UREASE ACTIVITY AND THE COMPOSITION OF PROTEIN GROUPS IN SOYBEANS DURING OIL PRODUCTION

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It is known that soybeans contain antinutritive compounds. These substances are determined in practice by measuring trypsin inhibitor or urease activity.

A comparative study of the two methods was carried out and a contradiction was discovered. It was established that during processing trypsin

inhibitor concentration decreased gradually while urease activity was lowered suddenly. The difference seems to be due to the fact that the changes as caused by heat in the antinutritive substances cannot be followed up unambiguously by urease activity measurement based on differences in pH.

On measuring urease activity by a new method involving titration, it was proven that the change in enzyme activity follows the same pattern as that of the trypsin inhibitor.

DETERMINATION OF THE GLUCOSE CONTENT OF PAPRIKA BY MEANS OF ENZYMES, A NEW ASPECT IN THE THEORY OF BROWNING

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The characterization of the colour of ground paprika by an objective numerical value is a necessity. The brown tint has a disturbing effect upon the colour. The main chemical components were studied to establish which of them, beside the carotenoids, carry the red and yellow colour and which of them may play a part in the development of the brown tint. It is known from the literature that browning is caused by caramelization of the sugars during processing. Recently it was established that the fibres are responsible for the brownish tint.

In the first step in studying this problem, the composition of the sugar content of ground paprika was established. Beside a small proportion of saccharose, glucose and fructose were detected in larger quantities, fructose being the predominating sugar. For the determination of all three sugars, enzymatic analyses were adapted. Glucose and fructose were determined by *Boehringer*'s UV test containing hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase. Saccharose was determined also by the *Boehringer*-manufactured UV test kit, containing hexokinase, glucose-6-phosphate dehydrogenase and invertase. In the ground paprika samples analysed, the proportion of the three sugars was as follows:

Glucose	1.32–3.37%
Fructose	5.18–9.28%
Saccharose	0.33–2.10%

Thus, potentially, the occurrence of caramelization during processing is possible. By chemical analysis, the presence of measurable amounts of

the degradation products of sugar upon caramelization could not be proved.

The protein content of ground paprika is about 17–18%. Many authors have shown that the protein and carbohydrate contents of foods may react even in the dry state to form indigestible compounds of brown colour. These compounds get bound to the cellulose skeleton and cannot be washed out. The reaction is accelerated by the moisture content and heating. The iron content is of the same effect. These three components are always present in paprika and ground paprika, although in varying amounts. From this, the conclusion was drawn that the rapid browning of ground paprika during storage might be caused by the *Maillard* reaction.

Thus, the technology as applied at present has to be changed. To protect the quality of the raw material, cold storage has to be given preference.

MODIFICATION OF THE COMPOSITION OF THE PLASMA MEMBRANE OF YEAST BY ADDING ERGOSTEROL AND POLYENE FATTY ACIDS

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In the growth and metabolism of yeasts used in various industries (brewing, alcohol industries, baker's yeast, enology) lipids play an important role. The composition of lipid membranes and depot lipids changes with the environment: under anaerobic conditions, they are not capable of synthetizing sterols and multiunsaturated fatty acids. Experience has shown the sterols (squalene, ergosterol, lanosterol) substantially change the fluidity of the plasma membrane. Under anaerobic conditions, in place of the long-chain unsaturated fatty acids, fatty acids of shorter hydrocarbon chain are synthesized.

Under anaerobic conditions, the plasma membrane of yeasts may be modified by adding lipids. An assay was made to add, under anaerobic conditions, phospholipids and ergosterol-fatty acid esters, forming the major part of the plasma membrane of brewer's yeast *(Saccharomyces cerevisiae*, or *carlbergensis)*. It was found that the polyene fatty acids and ergosterol in free (non-esterified) state become easily integrated into the membrane, while triglycerides and phospholipids have a much lower effect.

By adding long-chain, unsaturated fatty acids to the wort, the formation of short-chain fatty acids characteristic of beer may be retarded. This is important because C_6–C_{10} fatty acids influence disadvantageously the aroma

and volatile components of beer. Influencing fatty acid formation is particularly important for the so-called enzymic brewing, where the amount of oils and sterols gained from germinated malt is lower.

TOCOPHEROL CONTENT IN HUNGARIAN VEGETABLE OILS

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Vegetable oils contain different amounts of tocopherols.

The tocopherol content and the amount of tocopherol derivatives in the most important Hungarian vegetable oils (sunflower, rape seed, soya oil) were investigated. Results are given in the paper.

Investigations were extended over the (quantitative and qualitative) changes in tocopherol content of sunflower seed oil during processing, storage and in the course of oxidation.

INTERPLAY OF THE PIGMENT, OIL AND MOISTURE CONTENT IN THE DEVELOPMENT OF THE COLOUR OF GROUND PAPRIKA

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Mathematical correlations of pigment, oil and moisture content in the colour visually perceived or instrumentally established in ground paprika, have not yet been described in the literature.

In view of the computer-directed production of ground paprika it was, however, important to establish the mathematical relationship between changing chemical components and the instrumentally measured colour.

In order to resolve this problem, a model series of ground paprika samples of homogeneous granulation and predetermined pigment, oil and moisture content, altogether 23 samples, were tested in a MOMCOLOR apparatus and their X, Y and Z values were determined.

Results were evaluated by regression analysis. For each of the three colour components, separate fitting to the following polynome appeared to be expedient.

$$Y' = b_0 + \Sigma\, b_i x_i + \Sigma\, b_j x_k x_l$$

$$i = 1 \ldots 4 \quad j = 5 \ldots 10$$

$$k = 1 \ldots 3$$

$$l = 2 \ldots 4$$

where x_l is, in turn, the pigment content, expressed in g kg^{-1} or the proportion of red to total pigment content, or the oil content or the moisture content in %.

Series $x_k x_l$ were accounted for because it was established in the course of preliminary experiments that the effect of the individual components depends on the level of the other factors.

Values b_0–b_j of the function were determined by regression analysis based on the least squares method. Then the error of fit, the error of the estimated values of the parameters and the estimated significance tests were calculated.

Determination of the multiple correlation coefficient (R) showed the extent of dependence of the independent variable (the given colour component) on the interaction of the independent variables. The effect of the total pigment content on the values of X, Y and Z colour components was successfully proven at $P = 0.1\%$ significance level, and the interaction of total pigment content and oil content, as well as of total pigment content and moisture content at $P = 0.5\%$.

A close correlation was found between the chemical characteristics and the differences in colour related to the standard sample. This was proven by the value $R = 0.988$ in the case of $n = 23$ samples.

By means of the equations obtained for the colour components on one hand and the colour differences on the other, given the chemical composition prior to milling, the colour components of the expected ground paprika, or the ΔE colour differences in relation to the given standard sample, may be predicted.

By changing the composition of the semiproduct expediently, the difference from the standard sample may be kept within the given tolerance.

PHYSIOLOGICAL EVALUATION OF FOODS

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In developing more valuable foods, it is important to know how far the physiological requirements of the consumer are filled in by the planned composition.

The nutriment-energy index system was developed to solve this problem. The system is constructed on the principle that the essential nutriment requirement of the organism changes with the energy requirement.

The nutriment-energy index (NEI) is formed by dividing the quantity of each nutriment (N) (expressed in g, mg or μg) by its energy value (E) expressed in MJ:

$$NEI = \frac{N}{E}$$

The physiological nutriment-energy index (PNEI) is obtained by dividing the daily nutriment requirement (DNR) of a grown-up person, or the requirement of any diet (expressed in g, mg or μg) by the daily energy requirement (DER) expressed in MJ.

$$PNEI = \frac{DNR}{DER}$$

If the nutriment-energy indices are divided by the corresponding physiological nutriment-energy index, the physiological nutriment quotient is obtained:

$$PNQ = \frac{NEI}{PNEI}$$

PNQ stands for the relation of the nutriment mass to unit energy of the food studied and the physiological nutriment requirement related to unit energy.

If the physiological nutriment quotient is multiplied by hundred the percentage physiological nutriment supply is obtained:

$$PNP\ \% = PNQ \cdot 100.$$

If the PNQ value of an essential nutriment is 1, or its PNP % is equal to 100, the amount of the nutriment in the food satisfies the related requirement.

NUTRITIONAL EXPERIENCES RELATED TO THE MILK PROTEIN CONCENTRATE "SPORTROBI"

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The manufacturing technology of a concentrated milk protein, easy to digest, was developed at the Central Food Research Institute. The product is manufactured in the Milk Processing Plant, County Vas, since 1979 and at present is sold to hospitals and sport clubs in 10 kg bags under the name

Sportrobi. The product belongs to the category: new food. Its composition is as follows:

protein	84%
moisture	6%
ash	5%
lactose	2%
fat	3%

The product was tested in many different fields. Primarily it is useful where a high amount of protein or carbohydrate-deficient protein has to be consumed. It is suitable to satisfy the very high protein requirement of sportsmen and for the nutrition of persons in low health or after operation or suffering from diabetes, or from burn.

The product may be consumed added to various foods, or as a drink or pudding.

BIOGENIC AMINES IN THE BY-PRODUCTS OF THE MEAT INDUSTRY

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The processing of the by-products of the slaughterhouses and of confiscated animals, in order to satisfy the ever increasing protein feed requirement of animal husbandry, is an important task.

Since the raw materials of the protein meals of animal origin are frequently decayed and in a state of breaking down, it seems expedient to follow up the changes of certain characteristic components. It is of particular interest to find out the physiologically effective amine content of the products, dependent on the raw material and on the technology applied.

The study was aimed at some substances belonging to the biogenic amine compounds of the amino acid derivatives of feed meals of animal origin.

Histamine content was determined by ion exchange chromatography or by fluorometry. To determine free histamine, PCA extract purified by different methods, was used. Total histamine content was determined from a HCl hydrolysate.

By fluorometry, the histamine content of the samples amounted to 0–20 mg per 100 g protein meal. The highest value was obtained in fish meal. To detect further biogenic amines, the samples were chromatographed on a *Fixion* layer. The chromatograms were evaluated on a *Telechrom Videodensitometer.*

The following compounds were identified in the samples: tiramine, tryptamine, putrescine, cadaverine and histamine. In addition, one or two more ninhydrin-positive compounds were detected, however, not identified.

The changes occurring in the course of processing in the biogenic amines were modelled by the diluted aqueous solutions of standard amines at various pH values.

RESULTS OF MEASUREMENTS OF THE BASIC LEVEL OF RADIOACTIVITY IN THE ENVIRONMENT OF THE PAKS NUCLEAR POWER PLANT

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The control of the environment of the nuclear power plant under construction is carried within a region of 30 kilometres. Foods, raw materials of foods and other substances belonging under the competence of the Ministry, specified by the Law of Environmental Protection, were regularly and systematically investigated during the period 1977 to 1979. Specifications for sampling, and analysis were cross-checked with those of the network of national control.

It is unambiguously shown by measurements carried out hitherto that changes in the contamination of air caused by nuclear weapons tests seriously affect the artificial radioactivity in the air. For the control of the environment of the nuclear power plant, this fact means that the effect of two sources of contamination have to be detected side by side. Thus, besides surveying the radioactivity around Paks, a further aim of the study was to provide data, directly comparable to the national level of activity of the same period.

Due to the evenly distributed radioactivity all over Hungary, analysis of a desirable number of samples facilitates the separation of the contaminations of general or local origin in case a new source of contamination emerges.

INVESTIGATION OF EXTRANEOUS SUBSTANCES IN WINE FROM THE CONTAMINATED ENVIRONMENT

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Extraneous matter may get into wines from the contaminated environment by the following ways:

– Plant protecting agents settling on grapes and getting access from there into the wine because of non-observance of the prescribed waiting time or of

the concentration of the spray. In wines, residual fungicide agents have mainly to be accounted for. Lately, a case of contamination with a dithiocarbamate-type fungicide occurred leading to protracted fermentation and off-taste. The undecomposed residues and their derivatives were detected by thin-layer chromatography and spectrophotometry.

– Contamination originating from contaminated air, particularly in the vicinity of chemical plants. Air contamination may be caused by the defect of some technological equipment or simply by human carelessness.

In wines originating from such districts, a wide range of organic chlorine compounds was found. In addition, a great number of phenol and benzene derivatives was detected.

In one case contamination was not caused by a factory, but by the application of a herbicide from an agricultural helicopter to a near-by corn field.

Because of the unfavourable wind-direction or the mixing effect of the rotors, the herbicide got onto the grapes.

Air contamination was caused by the burning of a large quantity of discarded plastics. Pyrolysis products formed during burning were detected in the wine.

Contaminations getting into the wine from the storage containers are also considered environmental contaminants.

At one time, cement containers were lined with two-component synthetic resin instead of glass tiles. Here, because of careless work, styrene monomer got into the wine.

Beside the organic contaminants enumerated above, lately, heavy metal contamination was also discovered coming from plant protecting agents (copper, manganese, zink) or from exhauste gas (lead).

NUTRITIONAL SIGNIFICANCE OF NITRATES IN VEGETABLES AND THEIR PRODUCTS

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It has been known for the last three decades that methaemoglobinaemia in babies is caused by a high intake of nitrate or nitrite formed from nitrate by reduction. Earlier only the nitrate concentration in drinking water was considered responsible, however, recently an increasing number of papers holds the nitrates from food responsible for this disease.

The development of methaemoglobinaemia, probably due to physiological reasons, is mainly the disease of babies and small children. Since, according

to the literature, three quarters of the nitrates originating from food get into the organism with vegetables and their products, it was thought important to investigate the latter.

The nitrate content of vegetables varies within wide limits. Some of them accumulate a large amount of nitrate (beetroot, radish, spinach, kohlrabi and squash). In these vegetables, the nitrate content may reach, or even exceed, 1000–2000 mg kg^{-1}, while in others the nitrate concentration is lower by orders of magnitude.

The nitrate content of the processed vegetables was always lower than that of the fresh vegetable, and the nitrate content of canned products was found lower than that of the frozen products.

From the point of view of the nutrition of babies, the baby foods are of special interest. In considering their nitrate content, the proportion of the non-vegetable components added or the proportion of the individual vegetables in a mixture have to be taken into account. Generally, however, it may be said that a high nitrate concentration is found in those products which have a high nitrate content in the raw state.

The nitrate concentration in raw and processed vegetables and in the baby foods prepared of them fairly often reaches or even exceeds the level physiologically considered disadvantageous. Thus, it is desirable to reduce the nitrate content of the products by using the proper agrotechnique, variety and production technology.

METAL CONTAMINATION IN VEGETABLE OILS

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The knowledge of the metal content of vegetable oils and their products is indicated by foreign regulations and by the recently enacted Hungarian specifications. Metal contamination is of toxic and pro-oxidizing effect.

The iron, copper, nickel and lead content of oils was investigated by atomic absorption spectrophotometry.

The minimum quantity of iron, copper and nickel measured was 0.001 ppm while the minimum of lead amounted to 0.01 ppm.

It was established that the metal contamination in bottled vegetable oil does not exceed the maximum level permitted in Hungary.

APPLICATION OF THE ATOMIC ABSORPTION TECHNIQUE WITHOUT FLAME IN THE DETERMINATION OF COBALT IN CERTAIN FOODS

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Since there is a significant difference in the price of pork and beef liver, it is important for food control that there is a great difference in the cobalt content of the two (Tarján & Lindner, 1978). To identify or differentiate between the two raw materials of the meat industry, the following analytical method is suggested:

The weighed-in sample is first treated with nitric acid, then ashed at 400 °C. The ash is dissolved in diluted phosphoric acid, the solution is neutralized with sodium citrate and the cobalt (III) ions made to form a complex with 1-nitroso-2-naphthol (Koch & Koch–Dedic, 1974). The complex is then enriched by chloroform extraction and the cobalt content determined by the atomic absorption technique without flame. The calibration curve is plotted in the range of 0–0.25 $\mu g\ cm^{-3}$. The standard solutions are prepared in the same way as the sample solutions. The parameters of measurement were as follows: *Perkin-Elmer* Type 303 atomic absorption spectrophotometer, graphite cuvette Type HGA–70; *Perkin-Elmer* manufactured multielement cathode lamp (Mn, Cr, Co, Ni, Fe, Cu); heating current of the lamp: 30 mA; wave length: 240.7 nm; spectral slit width: 0.7 nm; rinsing gas: argon (1 $dm^3\ min^{-1}$) temperature program: (I) 100 °C 60 s, (II) 7P (3.4A) 90 s, (III) 24 A 30 s; no background compensation with deuterium is required. The calibration curve thus constructed is well utilizable in the concentration range studied but is not fully linear.

In the course of the atomic absorption measurement, the cobalt is reduced to metal in the cuvette and becomes contaminated with graphite causing a reduction in the melting point. An eutectic of 2.6–2.9% carbon content has a melting point of about 1320 °C (Gmelin, 1961). Reproducibility of the atomic absorption technique for cobalt determination:

	n	$\bar{x}$ ($\mu g\ g^{-1}$)	Coefficient of variation (%)
Pork liver	5	0.0099	14.1
Beef liver	5	0.0982	2.3

Naturally, the method may be used for other foodstuffs as well.

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SOLVENT RESIDUES IN VEGETABLE OILS

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A significant proportion of vegetable oils is gained by solvent extraction from the oil seeds. In Hungary, petrol is used as the solvent. The removal of the petrol residue from the oil is absolutely necessary, partly because of the fire and explosion hazard and partly because of the harmfulness of the residues.

A method was developed by the authors for the direct measurement of the petrol residue. The gas-chromatographic method was based on the experiences gained by the method of *Dupuy* and co-workers utilizing the same principle. The measurement of the solvent residue is carried out from the oil sample applied to the evaporating section of the gas-chromatographic column packed with silanized glass wool. Preparation of the sample is not necessary. Evaluation is carried out from a calibration curve on the basis of the integrated areas of the chromatogram.

The method is suitably reproducible for the determination of solvent residues above 10 ppm with a coefficient of variation of $\pm 5.2\%$. The method used at present in Hungary, based on the measurement of the change in mass during drying, enables the determination of more than 500 ppm solvent residue, only.

The technique was used for the analysis of residue in raw and refined vegetable oils. Raw oils contained 500–2000 ppm petrol. This value satisfies the specifications for raw oils. In edible oils, the residue was below 10 ppm, because it could not be detected. The utilization of the method in practice proved to be satisfactory.

STUDY ON THE BHT CONTENT OF EDIBLE FATS AND OILS BY A RAPID GAS CHROMATOGRAPHIC METHOD

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The availability of an appropriate qualitative and quantitative analytical method is a precondition of granting permission for the use of food additives.

Of the food antioxidants used in Hungary 3,5-di-terc-butyl-4-hydroxy-toluene (BHT) is the most important. The methods described in the literature for the determination of BHT are not sufficiently selective and their sensitivity is not satisfactory, either. With the usual chromatographic techniques, the requirement for work and chemicals is high.

In Hungary, BHT is used mainly in edible fats and there is a potentiality to apply it in vegetable oils. For the purpose of control in these fields a rapid, gas-chromatographic BHT determination method of low chemicals requirement was developed at the Institute of Nutrition.

The procedure is as follows: 1 g of the sample is exactly measured and dissolved in 7–8 cm^3 of an ether—petroleum ether (1 : 1) mixture and brought up to 10 cm^3. Of this diluted solution, 2–5 μl are directly injected into the gas-chromatographic column.

In the first 6–7 cm of the column glass wool prepack is used for binding the non-volatile fats and oil residues. The retention times and the calibration curve are prepared with standard solutions and these serve for identification and quantitative determination. The glass wool, used as prepack in the column has to be regularly replaced. If the column gets contaminated it needs to be heated, or replaced.

The main gas chromatographic parameters: Apparatus: *Packard* 419 GC. Detector: flame ionization.

Column:	A 2 m × 2 mm i.d. glass	B 2 m × 2 mm i.d. glass
Packing	*Chromosorb* P 100–120 mesh 20% DC–200	*Supelcoport* 100–120 mesh 10% SE–30
Column temperature	180 °C	160 °C
Carrier gas (N_2)	40 cm^3 min^{-1}	30 cm^3 min^{-1}
Retention time of BTH	14.4 min	6.5 min

The detectable minimum quantity of BHT is 2 ng, the detectable limiting concentration: 4 mg kg^{-1}. The relative error of the method is 0.5% for sunflower oil and lard, containing 100 and 20 mg kg^{-1} BHT, respectively. Recovery: 96–100%, 98% in the average. The method is suitable for the detection of BHA, too.

In model storage experiments the dynamics of BHT decomposition and the sensory characteristics of soybean oil were examined. Unfavourable storage conditions were not compensated by BHT, even at higher antioxidant concentrations. During the last year and a half, the Institute and the competent health authority examined 130 edible lard samples taken from the trade. In the samples which contained a detectable amount of BHT (55%), the level was either near the limit value (200–70 mg kg^{-1}) or at the lowest level (4–20 mg kg^{-1}). The lowest concentrations are probably not coming from the additive, but from tissue residues of the BHT consumed by the pigs in their diet. Thus, the BHT applied in animal husbandry forms partly a source of human exposure and it has to be taken into account in the wholesomeness evaluation of the additive.

NUTRITIONAL EVALUATION OF SWEETENING AGENTS NOT PROVIDING ENERGY

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The systematization of sweetening agents shows that to replace saccharose, both energy-providing and energy-nonproviding agents are used. It would be the task of the latter to ensure the hedonic value of foods, particularly for those suffering from diabetes and obesity, without entering the intermediate metabolism. Some of the non-sugar type compounds, though providing hardly any energy during metabolism, similarly to glucose, affect the carbohydrate metabolism and increase through the insulin effect excessive fat deposition in diabetics. These types of compounds are the peptides, oligopeptides, *e.g.* miraculin, monellin, thaumatin, aspartame, d-6-chlorotryptophan.

Because of the health hazards in relation to cyclamates and saccharin, in intensified research the dihydrochalcones came to the fore. By a Hungarian patent, it would be possible to manufacture from some dihydrochalcone type compounds sweeteners devoid of detrimental effect on the metabolism at a price not higher than that of sugar. This new sweetener was found suitable to be used in canned fruit and non-alcoholic drinks.

The enclosed Table serves with data on the efficiency of various sweeteners and on their toxicity.

Efficiency and expected consumption of different sweeteners at a daily sugar consumption reduced to half

Sweetener	Degree of sweetness (how many times sweeter than saccharose)	Equal to the sweetening capacity of 50 g sugar, mg	Notes on toxicity
Aspartame	160	312	Must not be given with Na glutamate
D-6-chlorotrypthophan	1300	38	Proved satisfactory in animal experiments
Monellin	2000	25	Non-toxic
Thaumatin	1600	31	Non-toxic
Neohesperidine dihydrochalcone propane sulfonyl derivative	1500	33	Non-toxic
Neohesperidine dihydrochalcone ethanolsulfonyl derivative	700	71	Non-toxic
Neohesperidine dihydrochalcone (Florida, USA)	1200	42	Non-toxic
Glycirrhizin	50	1000	Good
Cyclamates	35	1429	ADI value = 300 mg
Saccharin	500	100	ADI value = 180 mg

STABILITY OF ZEARALENONE WITH RESPECT TO THE POSSIBILITY OF DETOXICATION

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Under Hungarian climatic conditions, *Fusaria* are frequently encountered in the microflora of grain. Under temperature conditions advantageous for their growth, these fungi may produce metabolites toxic for men and animal. One of these metabolites is zearalenone (F-2 toxin).

It was established by derivatography and gas chromatography that the pure toxin is not damaged by heat up to 200 °C.

Model experiments were carried out with maize infected with *F. graminearum* (of a zearalenone concentration of 6.5 ± 0.68 ppm) in order to find out how the toxin content of maize is affected by chemical treatment. On steeping the maize with NH_4OH, NaOH, Na_2CO_3, "ultra" detergent, $Ca(OH)_2$ in aqueous solution of various concentrations, the toxin content was reduced by 20–80%. In the solution used for treatment, after appropriate neutralization, 70–90% of the toxin could be recovered. The heat resistance of the toxin was tested at 120 °C. No change was observed in the quantity of the toxin after a 4 h treatment at 120 °C.

The kinetics of toxin decomposition upon combined heat and chemical treatment was also investigated. A 100% decomposition of the toxin occurred upon treatment for 8 h under reflux with 1 *N* NaOH solution.

The effect of an oxidizing agent (1% aqueous H_2O_2 solution) was also studied. Upon treatment with the cold solution, the quantity of the toxin did not change. In a 1 : 1 (w/w) combination of Na_2CO_3 and toxin, when studied in the derivatograph a slight loss of weight was observed in the toxin between 165 and 220 °C. The substance formed during this treatment was investigated by gas chromatography. It was eluted at a temperature higher than the standard, but it could not be identified by MS test. When the reaction was carried out at isothermic conditions (180 °C) the quantity of the unknown compound first increased, then diminished and the reaction mixture turned into an insoluble black mass. On investigating the unknown compound by IR-spectrophotometer the intensity of the resorcylic OH' groups was reduced and an absorption peak, indicative of the ether group, appeared. It may be assumed, therefore, that the changes relate to polimerization.

ZEARALENONE IN BEER

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The major part of our food may be contaminated by moulds and their metabolites. The toxic metabolite of greatest importance from the view-point of human health may get into the food by the following ways:

– by consuming the contaminated food, *e.g.* sweet maize,

– by consuming food prepared with contaminated raw material, *e.g.* bread, apple juice,

– by consuming the metabolites after biological or microbiological change, *e.g.* beer, milk, meat.

The aim of this study was the investigation of the toxin in food after microbiological change.

Zearalenone may get into the beer in the following ways:

– the barley used for malting contains toxin,

– the toxin is introduced with another additive of brewing, *e.g.* maize,

– during the germination of the barley, the conditions promote the growth of *Fusaria*.

The toxin is determined in beer by the following method:

Five-hundred cm^3 of beer are clarified by *Carrez*'s clarifying agent ($K_4Fe(CN)_6$ and $ZnSO_4$), then extracted with chloroform. The organic phase

containing the toxin was evaporated to dryness and the residue dissolved in 2 cm^3 benzene. The benzene solution was injected into a 10×1 cm column of *Kieselgel*-60 packing. Elution was started with 20 cm^3 benzene and continued with a 9 : 2 mixture of benzene—acetone. The eluate was evaporated to dryness. To the dry residue 100 mm^3 TRI-SYL BT reagent were added. After a reaction time of 10 min 1 mm^3 was injected onto the gas chromatograph. Parameters: apparatus: *Packard* 7000; column: 16 mm×0.25 mm *(i.d.)* SE-52 glass capillary; temperature of the thermostat: 130–260 °C, 3°C min^{-1}; of the injector: 210 °C; of the detector: 240 °C; carrier gas: H_2.

In this study, the extent of the signal in the region 10 ng–1 μg was in linear relation to concentration. The method was suitable for use in the range of 10 ppb–10 ppm toxin. In the case of 100 ppb toxin content percentage recovery is 70%, the coefficient of variation $r = 12.7\%$ (n = 10).

In Hungarian beer commercialized during February and March, 1980 in Budapest no toxin was found by the above method.

EFFECT OF ADDITIVES AND TOBACCO SUBSTITUTES UPON BURNING OF CIGARETTES

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The burning characteristics of cigarettes are important not only from the point of view of consumption value but also from that of health, since the burning rate affects the amount of tobacco burnt during puff intervals and during puffs and, thereby it affects the smoke yield.

The burning rate of cigarettes is determined by the composition of tobacco, however this may be regulated by certain factors:

quality of the paper,

characteristics of the product,

additives and substitutes used in manufacturing.

The effects of the last two factors were examined.

– The effects of additives:

a) Of the sauces used (in the proportion of 3% as customary) diethylene glycol, propylene glycol and sorbitol reduce the burning index, while invert sugar and butylene glycol cause no change.

b) Of the additives used to improve burning potassium carbonate, nitrate and citrate proved effective for oriental tobacco mixtures when added at a rate of 2.5% (w/w). In *Virginia*, blend and dark type mixtures they were effective only with *Virginia*.

– The effects of tobacco substitutes:

a) Reconstituted tobacco increases significantly the burning rate of tobacco even in a proportion of 5%. The correlation between burning rate and reconstituted tobacco is of the saturation curve character.

b) The *Cytrel* tobacco substitute substantially increases the burning index. The increase is in linear correlation with the *Cytrel* content in the range studied.

THE EFFECT OF PLANT PROTECTING AGENT RESIDUES IN TOBACCO UPON HEALTH

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Since the consumption of tobacco differs basically from the consumption of foods, plant protectant residues have to be studied from a specific aspect. The limit values of protecting agent residues have to be specified for the product reaching the consumer on the basis of the quality and quantity of their pyrolysis products.

The pyrolytic decomposition of dithiocarbamate and maleic hydrazide residues was investigated, the former in order to modify the limit value and the latter in order to get permission for its use.

The standard substances were studied in modelled pyrolysis experiments and the smoking conditions of cigarettes under appropriate conditions were examined by instrumental analytical methods.

On the basis of the results, the limit value for dithiocarbamate was raised from 3 ppm to 25 ppm, while the waiting time was reduced from 21 days to 5 days. Permission is in the course of being granted for the use of plant protecting agents containing maleic hydrazide.

ANALYSIS OF THE PHYSIOLOGICALLY ACTIVE, VOLATILE COMPONENTS OF CIGARETTE SMOKE

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In view of striving for a healthier way of life and the increasing propaganda against smoking, it is necessary to try and reduce the harmful physiological effects of smoke. The reduction of the two most important tobacco smoke particles: tar and nicotin has already been resolved.

Of the tobacco components in gaseous phases, the following constituents of physiological importance were investigated with a view to the possibility of their reduction: HCN, oxo compounds, nitrogen oxides ($NO_2 + NO = NO_x$) and CO.

The HCN, formaldehyde, acetaldehyde and the acrolein contents of cigarette smoke exert a ciliastatic and ciliatoxic effect.

Nitrogen oxides are supposed to play a role in the formation of cancerogenic substances. The danger of CO lies in its haemoglobin-binding capacity. HCN was determined potentiometrically using a cyanide-selective electrode. Active carbon containing filters proved to be efficient in its reduction.

Two methods were developed to determine the oxo compounds. Their total amount was determined by colorimetry, while individually they were determined by gas chromatography. To bind them, filters of different composition proved to be selective.

NO was determined by spectrophotometry. The method is based on the specific colour reaction of NO and NO_2. It may be reduced in the smoke by the use of high-porosity paper, perforated cigarette and tipping paper. Certain filter additives seem also to promise good results.

For the determination of CO an apparatus based on infrared principle, was used. CO cannot be reduced by filters, however by diluting the main stream and the use of perforated cigarette and tipping paper and high-porosity paper and by improving the conditions of burning it may be considerably reduced.

The results achieved in reducing the harmful compounds have in practice been utilized. Up-to-date filters, perforated and high-porosity papers have already been introduced in the course of product development in the tobacco industry.

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COMBUSTIBILITY OF TOBACCO AS A FUNCTION OF ITS CHEMICAL COMPOSITION AND PHYSICAL CHARACTERISTICS

K. Arany-Füzesséry

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Combustibility of cigarettes is important not only from the point of view of sensory value but also from the health aspect.

Combustibility depends on the quality of the tobacco used and the cigarette paper. The combustibility of tobacco is affected by the variety, chemical composition, physical characteristics and growth conditions of the tobacco.

The burning capacity of tobacco generally cultivated in Hungary and taken from different areas was studied as a function of its chemical composition. Positive and negative correlations were established by linear regression and correlation calculation.

On the basis of data in the literature the relationship between combustibility and differential thermoanalytical characteristics of tobacco was investigated. The combustibility of tobacco could be assessed by the **L** index representing the distance between the minimum and maximum of the exothermic section of the DTA curve plotted by the *Derivatograph*.

The combustibility of tobacco affects its sensory value and is important from the point of view of health. The rate of burning and the temperature have a significant effect on the biologically active components of the smoke formed and on the proportion of individual components.

Investigations carried out hitherto show unambiguously that correlation exists between the pyrolysis temperature of cigarette glow and the amount of carcinogenic substances formed.

The combustibility of cigarettes depends mainly on two factors: the combustibility of the leafcut and of the cigarette paper. Other factors affecting combustibility are: the composition of the product, its physical characteristics and the quality and quantity of additives used in the leafcut.

The main factors in determining the combustibility of cut tobacco are the characteristics of the tobacco. A tobacco may be considered of good burning quality if ignited with a glowing device it burns without flame and glows for a long time. Glowing capacity may be characterized by the time and burning rate.

The combustibility of tobacco was investigated by a great number of researchers.

The papers published in the foreign literature on this subject are the result of research continued during long periods, perhaps several decades (Chouteau, 1966; Merker, 1970; Albo, 1974; Marcelli, 1974).

In Hungary this subject was studied many years ago (KOSUTÁNY, 1882; CSERHÁTI, 1893; BENŐCS, 1943).

On the basis of the data in the literature it may be said that the combustibility of tobacco is influenced by its variety, chemical composition, physical characteristics and conditions of cultivation. Beside the above, the mode of drying and the technology of curing also affect combustibility.

The available information, however, differs on many points. This is due probably to the fact that the investigations were carried out with tobacco grown under very different conditions, by different cultivation methods, under different climatic conditions and environmental influences.

A subject on which the various authors agreed is the positive correlation established between the potassium content of tobacco and combustibility. The opinion of the researchers agrees also on the negative effect of the chloride content. It was recently established that the advantageous effect of potassium asserts itself mainly when it is bound to organic acids. It is less effective in the form of mineral salt.

As regards other inorganic components and the organic ones, opinions are divided. However, it may be said that protein, resins, sugars, starch and cellulose exert a disadvantageous effect inasmuch as they cause the tobacco to burn with flame. The negative influence of resins and volatile oils may be explained by their capacity to produce in spite of their high carbon content easily inflammable volatile components, at slight heating.

Certain relations were discovered also between the combustibility and the physical characteristics, *e.g.* the structure of the leaves. It was observed that thin, fine leaves glow better than those of rough thick structure. However, the leaf structure as a measurable characteristic, is expressed in different terms by the different authors (PYRIKI & PHILIPP, 1955; PETERSON & TIBBITS, 1963; IVANOV *et al.*, 1973).

In the course of their differential thermoanalytical (DTA) work, SHIMADA and co-workers (1971) found a correlation between the DTA curves and the burning characteristics of tobacco. In their report, they established an index quantitatively reproducible, the so called **L** value. This represents the distance between the minimum and maximum of the exothermic part of the DTA curve in mm and is related to the combustion properties.

The present study was carried out to find correlations between the chemical components of tobacco and its burning period. Particular attention was paid to the relationship between the **L** value as determined on the basis of the DTA curve, and the burning period.

1. Materials and methods

Tobaccos of different variety and place of growing, generally cultivated in Hungary, corresponding to different quality classes were studied. Those of different combustibility were selected. A few foreign varieties were also investigated for purposes of comparison. Burning period was established with leaf-cuts of predetermined size (50 ×100 mm). The period between the removal of the device used for ignition (glowing cone or spiral) and the total extinction of the whole glowing area is expressed in seconds. Results were evaluated according to MERKER (1968) because this have shown a non-Gaussian distribution to prevail.

The extreme values exert a strong influence on the arithmetic mean, therefore, an improved mean value (**Z**) calculated on the basis of the correlation as given by the above author, is considered the burning period.

Derivatography was carried out with the parameters given in Table 1, on the *Paulik-Erdey Derivatograph* of this institute (manufactured by the HUNGARIAN OPTICAL WORKS [MOM]).

Table 1

Parameters of derivatography

Requirements	reaching 380 °C at a rate of 5 °C min^{-1}, retaining this temperature for 60 min
Inert substance	Al_2O_3
Tobacco weighed in	100 mg
Muffle furnace	1
Crucible	small Pt
Determination period (rotating drum)	200 min
Atmosphere	air
Beaker	quartz
Sensitivity	T: 500 °C TG: 100 mg DTG: 1/5 DTA: 1/10
Starting voltage	64
Pin series	1
Catch pin	4/29

The chemical components were determined by the methods used in the laboratory of our institute in routine analyses. The ash, the water-soluble ash alkalinity, the pH of the aqueous extract, the K, Ca, Mg, Cl, P and nitrate contents of tobacco were established. Further the water-soluble and volatile organic acids, volatile bases and total volatile base content, and other organic compounds like cellulose, starch, pectin, reducing substances (mono- and polysaccharides) were investigated and quantitatively determined.

2. Results and conclusions

The **L** values as read on the derivatograms and the **Z** values representing the burning period of tobacco are summed up in Table 2.

Table 2

Combustion properties and derivatographic data of tobaccos of different places of cultivation and quality classes

Variety	Place of cultivation	Burning period **Z**(s)	Derivatographic data **L** (mm)
Szabolcsi V. II.	Fülöp	15.5	24.0
Szabolcsi III.	Fülöp	5.5	24.8
Hevesi V. II.	Nyírbátor	5.3	26.0
Kerti V. II.	Nyíracsád	7.5	24.0
Kerti B. II.	Nyíracsád	4.5	24.5
Kállói V. II.	Aradványpuszta	13.5	24.0
Kerti III.	Nyíracsád	7.2	25.2
Sota 6605 V. I. II. Bale number: 9609	Levelek	8.5	31.0
Sota 6605 V. I. II. Bale number: 9599	Levelek	7.0	29.5
Sota 6606 B. I. II. Bale number: 17052	Levelek	2.9	31.5
Sota 6605 B. II. Bale number: 17060	Levelek	2.9	31.5
Sota 6605 B. II. Bale number: 17058	Levelek	2.2	28.5
Sota 6605 V. II. Bale number: 9668	Bököny	1.1	34.5
Sota 6605 V. II. Bale number: 9675	Bököny	1.6	30.5
Sota 6605 B. II. Bale number: 17305	Bököny	2.1	31.0
Sota 6605 B. II. Bale number	Bököny	2.3	32.0
Imported tobacco			
Harmenli from Bulgaria 1972		2.5	30.0
Albanese, Duhan IV. 1972		3.7	28.0
Virginia from North Korea IV.		7.5	25.0
Virginia from India 1973		2.6	30.5

The results were used in linear regression and correlation calculations. The data were used to construct the correlation diagram and to determine the equation of the regression lines for two variables:

$$\mathbf{L} = 30.95 - 0.558\,\mathbf{Z}$$

$$\mathbf{Z} = 32.87 - 0.913\,\mathbf{L}$$

where **Z** = burning period,

L = derivatographical index.

A more exact description of the relationship between the two variables is given by the resultant line traceable between the two regression lines. The equation of this line is the following:

$$\mathbf{L} = 33.5 - 0.83\,\mathbf{Z}$$

The correlation coefficient is $r = 0.714$, thus the correlation may be considered close.

The correlation coefficient was statistically tested. The critical r value, as given in the Table, at $n - 2 = 18$ degree of freedom for the probability level of $P = 5\%$ is $r = 0.4438$. The absolute value of r as calculated is higher than that of the critical value, thus it may be considered proven that r differs significantly from 0, thus variables **L** and **Z** are correlated.

On the basis of the above, it is possible to draw conclusions indirectly as to the combustibility of tobacco by derivatographic analysis.

As regards the quantity of chemical components determined in relation to leaf burn a wide range of values was found (see Tables 3 and 4). This is advantageous from the point of view of analysis.

Table 3

Combustion properties and chemical composition of tobacco variety Kállói cultivated at two different locations

Quality characteristics	Range at place of cultivation		Mean values	
	Levelek	Bökény	Levelek	Bökény
Burning period (*s*)	2.20 – 8.50	1.10 – 2.30	4.70	1.80
Raw ash (%)	17.00 – 23.80	14.70 – 27.50	19.30	19.50
Alkalinity in ash (0.1 *N* HCl $cm^3\,g^{-1}$)	2.24 – 3.44	0.43 – 1.54	2.88	0.95
Potassium (%)	2.45 – 3.12	2.30 – 2.85	2.89	2.67
Calcium (%)	3.45 – 4.87	3.10 – 4.21	4.04	3.65
Magnesium (%)	0.53 – 0.63	0.52 – 0.57	0.58	0.54
Chloride (%)	0.94 – 1.14	1.19 – 3.21	1.03	2.36
Phosphorus (%)	0.22 – 0.34	0.21 – 0.23	0.28	0.22
$(K^+ + Ca^{++})$-Cl^-	5.17 – 6.83	3.22 – 5.88	5.90	3.94
Cellulose (%)	14.92 – 19.42	14.52 – 15.04	16.29	14.76
Pectin (Ca-pectate) (%)	5.90 – 8.91	7.70 – 10.88	7.03	9.11
Starch (%)	0.72 – 0.95	0.80 – 1.16	0.83	0.98
Monosaccharide (%)	0.68 – 0.87	0.60 – 1.73	0.75	1.25
Polysaccharides (%)	0.84 – 1.22	1.07 – 1.29	1.05	1.16
Volatile organic acids (%)	0.70 – 0.97	0.68 – 0.95	0.86	0.86
Water-soluble organic acids (0.1 *N* NaOH $cm^3\ g^{-1}$)	0.12 – 0.14	0.12 – 0.14	0.12	0.12
pH of aqueous extract	5.97 – 5.99	5.70 – 5.78	5.98	5.73
Total alkaloid (%)	1.37 – 2.09	1.41 – 2.95	1.93	1.94
Volatile bases (%)	0.72 – 1.08	0.77 – 0.81	0.89	0.80
Total N (%)	4.28 – 6.24	4.66 – 5.24	5.68	5.12
Protein N (%)	2.30 – 3.19	2.33 – 2.80	2.74	2.57

Table 4

Combustion properties and chemical composition

Samples and their place of origin	Burning period (*s*)	Raw ash (%)	Alkalinity of ash (0.1 *N* HCl $cm^3\ g^{-1}$)	K (%)	Ca (%)
Szabolcsi V. II. (Fülöp)	15.5	23.3	5.30	2.81	3.80
Szabolcsi III. (Fülöp)	5.5	24.0	3.40	2.67	3.24
Hevesi V. II. (Nyírbátor)	5.3	16.3	2.75	2.71	2.91
Kerti V. II. (Nyíracsád)	7.5	23.9	4.32	2.20	2.73
Kerti B. II. (Nyíracsád)	4.5	22.4	2.05	1.75	4.55
Kállói V. II (Aradványpuszta)	13.5	22.5	5.19	2.75	4.40
Kerti III. (Nyíracsád)	7.2	20.9	2.93	2.84	3.52
Bulgarian Harmenli (1972)	2.5	20.4	1.50	2.87	4.04
Albanese, Duhan IV. (1972)	3.7	15.8	1.60	2.02	2.77
Virginia from North Korea IV.	7.5	15.3	3.86	2.73	2.53
Virginia from India (1973)	2.6	16.9	1.54	1.70	3.90

The effect of the various components on the combustibility of tobacco was established by mathematical statistical analysis and correlation calculations of the results.

By means of simple correlation coefficients, the individual components were found to be in direct or inverted relationship with combustibility as follows.

The correlation coefficient was found to be positive and of a value higher than 0.50 in the case of Ca, K, cellulose, alkalinity of ash and [K + Ca)–Cl]. It was negative for chloride, starch, pectin, water-soluble and total volatile organic acids and sugars.

Weak correlation was observed in the case of Mg, phosphorus and raw ash. No correlation was found for the other components.

In the case of the samples investigated, the nitrate content was found to relate mostly to variety.

of Hungarian and foreign tobacco samples

Mg (%)	Chloride (%)	Phosphorus (%)	Nitrate (%)	(K + Ca)-Cl	Water-soluble organic acid (0.1 *N* NaOH cm^3 g^{-1})	pH	Alkaloid (%)
0.64	0.13	0.33	0.14	6.48	0.105	5.90	1.33
0.58	0.18	0.34	0.14	5.73	0.100	5.68	0.90
0.43	0.22	0.40	0.02	5.40	0.105	5.66	0.69
0.71	0.13	0.18	0.01	4.80	0.100	5.59	2.06
0.74	0.33	0.23	0.08	6.60	0.100	5.97	1.52
0.39	0.18	0.47	0.02	6.98	0.110	5.75	1.17
0.58	0.12	0.28	0.06	6.22	0.075	6.24	0.65
0.77	0.50	0.21	0.13	6.41	0.165	5.15	1.18
0.59	0.15	0.26		4.64	0.150	5.12	0.48
0.42	1.31	0.35	0.02	3.95	0.190	5.07	1.05
0.87	1.78	0.22	0.08	3.72	0.140	5.21	1.78

Since the simple correlation is the measure of the relation between two variables, the results, irrespectively of the "complex" effects of other components, may be accepted only in first approximation.

The analysis of the individual components independently from the others is not sufficient for judging combustibility, because their effect is compensatory and complementary. It may be established unambiguously, however, that the combustibility of tobacco is in close correlation with its chemical composition. The correlation is, however, rather intricate, because it is affected by many other factors as well.

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FOOD FLAVOURING WITH β-CYCLODEXTRIN-COMPLEXED FLAVOUR SUBSTANCES

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β-Cyclodextrin composed of seven glucopyranose rings is able to form inclusion complexes with flavour substances of low molecular mass (Mmass 80–250). The molecules of active ingredients of almost all natural spices and flavours fit into this range. Advantages of their stable β-cyclodextrin complexes: constant composition, macroscopic and microbiological purity, decreased sensitivity to storage circumstances (heat, light, time) and stability to oxidation, polymerization, sublimation are known. Sensory analysis of normal and dietetic food has proved, that β-cyclodextrin flavour complexes are highly efficient substituents of natural spices both as single and mixed flavourings.

For food flavouring natural spices of plant origin are generally used. Some disadvantages of their application are: (i) unstable aroma content that depends on species, way of cultivation, processing; (ii) loss of aroma content on storage (volatility, oxidation, polymerization); (iii) unavoidable macro- and microscopic impurities.

One of the most promising possibilities of stabilizing aromatics, is their inclusion complex formation (molecular encapsulation) with β-cyclodextrin (Rogers & Whaley, 1962; Szejtli *et al.*, 1977; Szejtli *et al.*, 1979).

The molecular encapsulation of a number of compounds can be performed with β-cyclodextrins. This process often advantageously modifies various physical and chemical properties of the molecule encapsulated. This method is simpler and cheaper than most of the rest. The molecular structure of cyclodextrins renders them capable of including other molecules, as a result of which powderlike crystalline inclusion complexes are produced. This is the essence of molecular encapsulation.

Cyclodextrins and their complex-forming nature have long been known but cyclodextrins were available only as fine chemicals in small amounts at rather high prices – a fact that impeded their industrial utilization. As a result of intensive research during the past decade, the industrial production of β-cyclodextrin has become reality, its application in the pharmaceutical, food and chemical industries seems now therefore possible.

Molecules, or functional groups of molecules having appropriate molecular dimensions that fit into the cyclodextrin cavity, being less hydrophilic than water can be included into the cyclodextrin cavity if both components are dissolved in water. In aqueous solution, the slightly apolar cyclodextrin

cavity is occupied by water molecules that are energetically unfavoured and are readily substituted by appropriate "guest molecules" that are less polar than water (BERGERON *et al.*, 1977). The inclusion complexes formed are very stable, their water solubility is strongly reduced so they rapidly separate in crystalline form. The physical and chemical characteristics of the complexed molecules become significantly modified (BENDER & KOMIYAMA, 1978). Because covalent bonds do not form between the components under physiological conditions, the complex easily dissociates.

The structure of crystalline cyclodextrin complexes is not always identical with that of the complexes in solution. In dissolved state the guest molecule (or its corresponding group) is located within the cavity of cyclodextrin and the whole complex is surrounded by a multilayer hydrate hull. In the crystalline state, guest molecules are located not only inside the cyclodextrin cavity but also between the cyclodextrin rings, in the form of crystal lattice inclusion. At the same time, some of the cyclodextrin molecules include only water, consequently they are incorporated into the crystal lattice as water complexes. Therefore, the crystalline complexes are practically never of a strict stoichiometric composition and are also stable if the ring cavities are only partially saturated by apolar guest molecules.

It is simple to prepare inclusion complexes. The method most often applied is to stir or shake the aqueous solution (cold or warm, neutral or acidic) of cyclodextrin together with the guest molecule or its solution. After

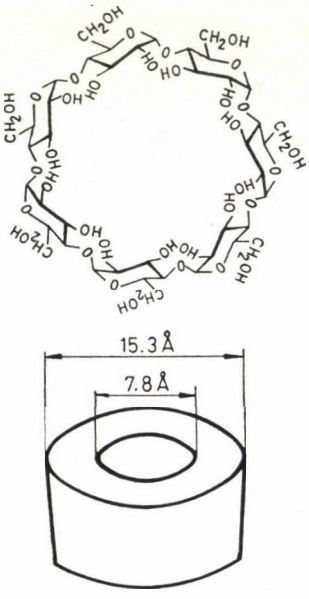

Fig. 1. Chemical and molecular structure of β-cyclodextrin

equilibrium has been attained, water is eliminated by freeze-drying, spray-drying or by any other convenient method. Most frequently the microcrystalline product is separated by filtration (CRAMER & HENGLEIN, 1957).

Ring size of β-cyclodextrin, composed of seven glucopyranose rings, makes possible the formation of inclusion complexes with food aromatics in the molecular mass range of Mmass 80–250 (monoterpenoids, phenylpropanes). Substances of higher molecular mass are also capable of complex formation if the molecule contains a side chain of appropriate size and shape that fits into the cyclodextrin cavity (Fig. 1).

Essential oils extracted from spices (onion, dill, tarragon, marjoram, garlic *etc.)* readily form stable crystalline inclusion complexes with β-cyclodextrin. These aromatics generally consist of a number of components. Using appropriate complexation technology, practically unaltered flavour composition can be preserved in the complex (Tables 1 and 2).

In addition to natural essential oils, also some synthetic compositions like smoke extracts can be taken into consideration for cyclodextrin inclusion complex formation.

Figure 2 illustrates the gas-chromatographic pattern of an original essential oil and its β-cyclodextrin inclusion complex. Flavour complexes in dry condition are very stable. Their oxygen uptake (measured by the *Warburg* technique) does not come as high as 10% of that of the free, non-complexed compounds. They only lose 25–30% of their aroma content after 24 h at 150 °C in vacuum while the pure spice aromatics evaporate completely under similar conditions (SZEJTLI *et al.*, 1979).

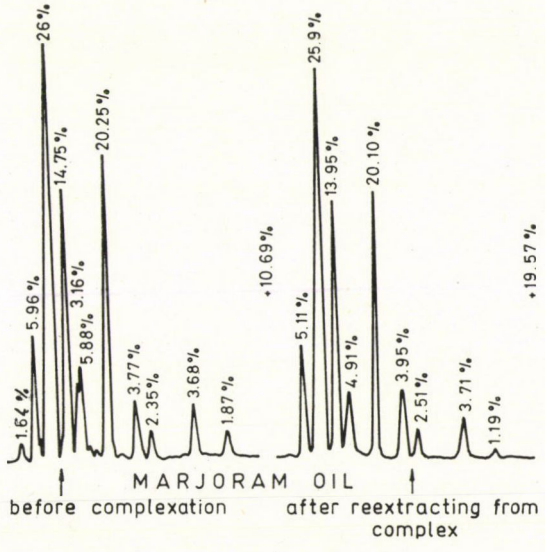

Fig. 2. Gas chromatograms of free and complexed marjoram oils

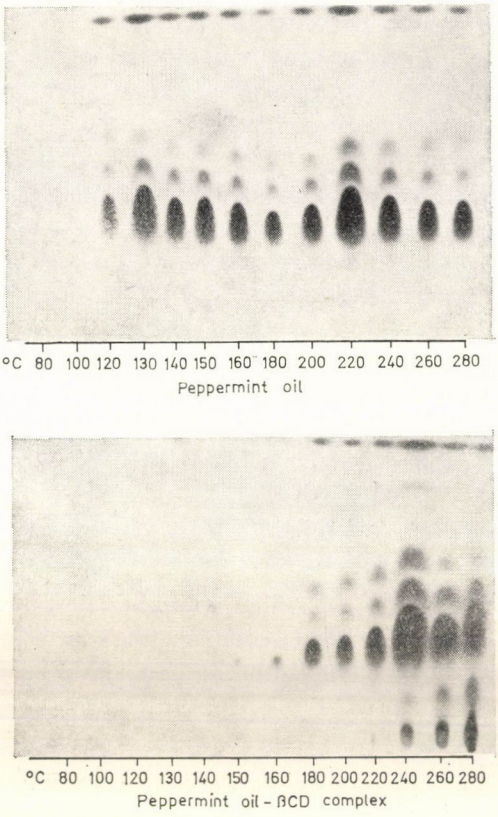

Fig. 3. Heat stability of peppermint oil and its complex by TAS technique. Developed on *Kieselgel G* plates, solvent: benzene–ethylacetate (90 + 10), vizualization: $SbCl_5$ in chloroform, heating at 110 °C (SZEJTLI *et al.*, 1979)

Heat stability is demonstrated in Fig. 3 applying pyrolytic thin-layer chromatography (TAS technique). Peppermint oil is volatile at 120 °C, while its cyclodextrin complex starts evaporating only at 180 °C.

Six months chronic toxicity of β-cyclodextrin was studied in rats up to 1.6 g kg^{-1} per day and 0.6 g kg^{-1} per day in dogs (MAKITA *et al.*, 1975; SZEJTLI & SEBESTYÉN, 1979). No signs of toxic effects were observed in respect of weight gain, food consumption or in clinico-biochemical values. Pathological checking of digestive organs, the central nervous system, the cardiovascular system and other organs have not revealed any sign indicative of toxicity. Orally administered cyclodextrin can be considered as a non-toxic substance. According to FAO Nutrition Meetings Report Series No. 46 A WHO/FOOD AD/70.36, in case of enzymatically modified starches, toxicological tests are not required. β-Cyclodextrin was also devoid of embryotoxic and gene-mutation-including effects (SZEJTLI & SEBESTYÉN, 1979).

Table 1

Some examples of β-cyclodextrin inclusion-complex-forming flavour substances (Data compiled from the literature)

Aroma components	Mmass	Approximative	
		length (nm)	diameter (nm)
allicin	162.27	1.2	0.5
allyl-isothiocyanate	99.19	0.7	0.4
diallyl-disulfide	146.26	1.2	0.5
anethole	148.20	1.0	0.6
benzaldehyde	106.12	0.9	0.6
benzyl alcohol	108.13	0.9	0.6
benzoic acid	122.12	0.9	0.6
borneol	154.24	1.1	0.7
cineol	154.24	1.0	0.7
citral	152.23	1.0	0.6
citronellol	156.26	1.0	0.6
cinnamaldehyde	132.15	0.9	0.7
cinnamic acid	148.16	0.9	0.7
eugenol	164.21	1.0	0.6
fenchone	152.23	1.1	0.7
geraniol	154.24	1.0	0.7
camphene	136.23	0.9	0.7
carvone	150.21	1.0	0.7
linalool	154.24	1.0	0.7
menthol	156.27	0.9	0.6
oenanthic acid ethyl ester	158.00	1.3	0.5
pelargonic acid ethyl ester	186.30	1.5	0.5
α- and β-pinenes	136.23	1.0	0.7
salicylic acid methyl ester	152.00	1.1	0.7
terpineol	154.24	1.0	0.6
thymol	150.22	0.9	0.6
vanillin	152.14	1.0	0.7

Products will be marketed by *Compack Packaging Enterprise*, Budapest. Toxicological documentation submitted to the *National Institute of Nutrition* (as the adviser to the relevant Hungarian Public Health Authority) was based on *per os* toxicological tests of β-cyclodextrin performed by *Chinoin Pharmaceutical Works*, Budapest. β-Cyclodextrin characterized by adequate quality requirements was considered as non-toxic substance by the *National Institute of Nutrition*.

Table 2

Essential oils that practically form β-cyclodextrin inclusion complexes of unchanged composition

No.	Essential oil	No.	Essential oil
1	basil oil	16	lovage oil
2	bay leaf oil	17	marjoram oil
3	bergamot oil	18	mustard oil
4	calamus oil	19	nutmeg oil
5	camomile oil	20	onion oil
6	caraway oil	21	parsley seed oil
7	carrot oil	22	parsley weed oil
8	cinnamon oil	23	peppermint oil
9	clary oil	24	rosemary oil
10	clove oil	25	sage oil
11	coriander oil	26	savory oil
12	dill seed oil	27	spearmint oil
13	dill weed oil	27	sweet cumin oil
14	garlic oil	29	tarragon oil
15	hyssop oil	30	thyme oil

1. Materials and methods

1.1. Materials

1.1.1. β-Cyclodextrin-flavour complexes. Crystalline aroma complexes were prepared as described earlier (SZEJTLI *et al.*, 1979). β-Cyclodextrin is a product of *Chinoin Pharmaceutical Works*, Budapest. The following flavour components (extracts) were obtained from KHV *(Enterprise for Cosmetics and Toiletry Articles*, Budapest):

garlic
thyme
dill
caraway
lovage
marjoram
mustard
rosemary
onion
smoke (Fumosal)

Aroma contents of β-cyclodextrin-flavour complexes were around 10%.

1.1.2. Natural spices and foodstuffs. To prepare foods flavoured with natural spices and β-cyclodextrin- flavour complexes raw materials and foodstuffs of commercial quality were used.

1.2. Methods

1.2.1. Food preparation. Foods were prepared according to the recipes of VENESZ and TÚRÓS (1964). The control samples were prepared from the same raw materials and ingredients except flavourings. Tables 3 and 4 show the amounts of natural spices and equivalent β-cyclodextrin-flavour complexes necessary to season the foods.

Sausages were produced according to the recipes of the *Manual of the Meat Industry* (LŐRINCZ & LENCSEPETI, 1973). Some hundred grams of meat products were spiced with β-cyclodextrin-flavour complexes. The amounts of complexes added were calculated as given in Table 5.

1.2.2. Sensory analysis – Triangular method and Paired comparison test. For testing, trained panelists of both sexes, aged 18–24 years were selected according to the standard ISO/DIS 3972. Triangular test was carried out with panels of 20 members as described in the international standard ISO/DIS 4120 and the Paired comparison test according to ISO/DIS 5495. Besides differentiation and identification also preference was biometrically evaluated using the supplementary tables of the standard.

1.2.3. Consumer tests. Three panels of 40 members each participated in consumer tests. Group 1 consisted of tasting experts recruited from administration and research institutes, Group 2 of producers and businessmen from the catering trade, Group 3 of housewives, intellectuals and manual workers.

2. Results

2.1. Application of flavour complexes in catering and the meat industry

Foods prepared with β-cyclodextrin-aroma complexes were unanimously preferred by panelists ($P = 99.9\%$) to those prepared with natural spices even if mixtures of complexed flavours were applied (stewed Savoy cabbage, spiced ewe cheese). Foods flavoured with β-cyclodextrin-flavour complexes have been found equally acceptable by all the three groups (more than 100 assessors). Less than 10% of the panel members objected to the uncommon flavours like lovage and tarragon but they also disliked the taste of these natural spices. Heat-treated sausages were compared with commercial meat products using the paired comparison test. The results of sensory analysis indicated, that by using β-cyclodextrin-flavour complexes, the most dangerous sources of impurity could be eliminated (natural spices) and the preparation of more highly flavoured foods became possible.

Table 3

Weight of spices and equivalent β-cyclodextrin-flavour complexes in 100 portions of different foods

Foods	Spices (g)						β-cyclodextrin-flavour complexes (g)*					
	garlic	onion	caraway	marjoram	mustard	dill	garlic	onion	caraway	marjoram	mustard	dill
Thick brown soup	—	—	50	—	—	—	—	—	22.5	—	—	—
Garlic sauce	200	—	—	—	—	—	10	—	—	—	—	—
Dill sauce	—	—	—	—	—	300	—	—	—	—	—	2.0
Meat pie with marjoram	—	—	—	13	—	—	—	—	—	2.5	—	—
Spiced ewe cheese	—	500	7	—	100	—	—	10.0	3.3	—	1.5	—
Stewed Savoy cabbage	60	—	7	15	—	—	3.3	—	3.3	3.3	—	—

* Calculated on the basis of the utilized amount of spice

Table 4

Amount of β-cyclodextrin-flavour complexes in foods that were found appropriate by sensory analysis (g in 100 portions)

Foods	Garlic	Onion	Caraway	Dill	Marjoram	Mustard	Tarragon	Lovage	Rosemary	Thyme	Smoke
Hors d'oeuvres with spiced ewe cheese	—	1.5	3.3	—	—	1.5	—	—	—	—	—
Hors d'oeuvres with ham	—	—	—	—	—	—	—	—	—	—	0.6*
Vegetable cream soup with lovage	—	—	—	—	—	—	—	0.45	—	—	—
Stewed Savoy cabbage	3.3	—	3.3	—	3.3	—	—	—	—	—	—
Meat fingers	—	5.0	—	—	—	—	—	—	—	—	—
Roast beef with dilled butter	—	—	—	2.0	—	1.6	—	—	—	—	—
Lettuce with tarragon	—	—	—	—	—	—	1.3	—	—	—	—
Meat balls with dill sauce	—	—	—	3.0	—	—	—	—	—	—	—
Patties English style	—	—	—	—	—	—	—	—	—	3.3	—
Hamburger, Italian style	—	—	—	—	—	—	—	—	3.0	—	—
String beans, Hungarian style	3.3	3.3	—	—	—	—	—	—	—	—	—

* For concentrated pickling solution

Table 5

Some examples of application of flavour complexes in the meat industry

Foods	Spice (g) to 100 kg product							β-cyclodextrin-flavour complex (g) to 100 kg product						
	garlic	onion	nutmeg	marjoram	caraway	coriander	mustard	garlic	onion	nutmeg	marjoram	caraway	coriander	mustard
Veronese	50	—	50	—	—	—	—	2.5	—	22.5	—	—	—	—
Csaba sausage	50	—	—	—	—	100	—	2.5	—	—	—	—	10	—
Gyula dry sausage	450	—	—	—	50	—	—	22.5	—	—	—	22.5	—	—
Liver sausage	—	3000	—	60	—	—	—	—	3.0	—	12	—	—	—
Liver mousse	—	2000	—	60	—	—	—	—	3.0	—	12	—	—	—

Table 6

Number of patients on different clinical diets given food flavoured with various β-cyclodextrin-flavour complexes

(Data of the INSTITUTE OF NUTRITION, Bratislava)

Spices	Type of diet					
	Protecting	Bile	Liver	Fat reducing	Diabetic	Salt-free
Caraway	8	8	5	15	—	2
Dill	28	18	15	—	2	15
Marjoram	6	5	5	—	2	—
Mustard	8	5	2	16	4	—
Garlic	40	31	23	16	12	12
Thyme	3	—	—	—	1	—

2.2. Application of flavour complexes in clinical nutrition

In addition to food industrial application, β-cyclodextrin-flavour complexes could advantageously be used in clinical nutrition as has been found at the *Department of Metabolic Diseases, Institute of Nutrition*, Bratislava (Bučko, 1979). Table 6 shows the number of patients on different clinical diets consuming food flavoured with various β-cyclodextrin-flavour complexes. None of the 307 patients complained of any malady symptom or unpleasant effects. Subjective repugnance has not been observed, not even in cases when in clinical diet flavours otherwise not applied had been used (mustard, garlic, thyme). Thus β-cyclodextrin-flavour complexes proved to be acceptable ingredients in clinical dietetic food preparations.

2.3. Other applications of flavour complexes in nutrition

Tea of poor quality can also be flavoured with β-cyclodextrins mixing various amounts of bergamot, jessamine, lemon or peppermint containing complexes to tea leaves. Either granulated aroma complexes are added to the content of the tea bags or master mixtures are prepared and mixed to tea leaves before packing. The aromatized tea samples have been preferred with high significance to original non-aromatized tea by both organoleptic scoring and triangular tests.

Preliminary examinations of dehydrated soups prepared with β-cyclodextrin-aroma complexes are also very promising, thus utilization of flavour complexes seems to be reasonable also in this respect.

3. Conclusions

In the course of our study β-cyclodextrins proved to have advantageous qualitative, technological and hygienic properties compared to natural spices.

Both single and mixed β-cyclodextrin-flavour complexes are generally white crystalline substances mixed advantageously with common salt or powdered sugar. They are devoid of microscopic and microbiological impurities, and can be stored without loss of their aroma content for years.

Advantages of β-cyclodextrin-flavour complexes can be summarized as follows:

- in households

  the fact that complexes can be added when the food has already been cooked makes correction of flavouring easier and widens the range of aromas. They can be stored without loosing their flavour content for a long time and do not need much room for storage;

– in catering
with the practical elimination of *in situ* processing of natural spices, requirements for manpower and storage space can be reduced. Also transport gets simpler. With the elaboration and application of appropriate flavour complex combinations, standard flavouring may be warranted;
– in clinical nutrition
consumption of high crude fibre containing spices and flavours can be avoided. In the complexed form, patients on clinical diet may consume even those aromas which they were not allowed to enjoy in the form of spices because of the high fibre content, microbiological impurities, and irritating effects of the latter *(e.g.* piperin);
– in the meat and canning industries
β-cyclodextrin-flavour complexes as stable compounds of constant composition can be applied to sausages and dehydrated soups without the danger of microbiological contamination.

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QUANTITATIVE DETERMINATION OF MILK AND SOYA PROTEINS IN MEAT PRODUCTS

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A suitable method was developed for the quantitative determination of milk and soya proteins added to meat products. The method is based on the densitometric measurement of milk and soya protein fractions separated by polyacrylamide gel electrophoresis and stained with Fast Green. Codenaturation of meat and non-meat proteins was considered by the use of an adequate standard. The minimal detectable concentration of both sodium caseinate and Promine D was 0.4%. The coefficient of variation of the method proved to be ±10% in the case of milk protein and ±15% in the case of soya protein.

Milk and soya proteins play an important role as ingredients in meat products because of their favourable technological properties (water and fat absorption and fat-emulsifying capacity). Their application is permitted in limited quantities in certain types of meat products in Hungary. Therefore, and further because differentiation of meat from non-meat proteins may be necessary also from nutritional and dietetic aspects, to possess a routine method for their quantitative determination is of primary importance. There are several methods described in the literature but these cannot be widely applied in our practice and/or as a routine method. Most frequently methods applied are based on immunoelectrophoresis or immunodiffusion (KRAACK, 1973; WU & INGLETT, 1974; GÜNTHER, 1974; BAILEY, 1976; BAUDNER, 1977; BAUDNER *et al.*, 1977). These methods are mostly reliable and accurate. The disadvantage lies in their being rather labourious and time-consuming and the preparation of the antisera may be problematic in some laboratories. In the case of heat-treated, cooked meats, detection can be carried out only by connecting characteristic fractions of fractionated soya protein to human or bovine albumin (BAUDNER *et al.*, 1977; GÜNTHER *et al.*, 1977). Applying this preparation to produce antiserum, immunochemical investigations may be carried out. The method of DE HOOG and co-workers (1970), based on the difference in relative densities, is suitable for the quantitative determination of 0.3–2.0% of non-meat proteins within ±20% margin of error. The quantitative measurement is carried out by nitrogen determination of layers of different relative density obtained by centrifuging according to the density gradient. Milk and soya proteins are distinguished according to their different selective solubility. The procedure is time-consuming and labourious.

FISCHER and co-workers (1976) determined the soya content of meat products by measuring canavanine [2-amino-4(guanidino-oxy)butyric acid] concentration. This amino acid is present in free form in a lot of different plants but does not occur in meats. Authors proposed this method beside electrophoresis, to obtain confirmative results.

FISCHER and BELITZ (1976) isolated the "2" band of soya protein by preparative polyacrylamide gel electrophoresis. This band was identified by its known amino acid composition.

The soya flour and soya groats content of products may be determined according to their hemicellulose content (LEE *et al.*, 1975) but this method is not suitable to detect either soya protein concentrates or isolates.

The following method proved to be expedient in the USA: a known quantity of titanium dioxide is added to the soya containing products and the soya content of different products is measured by the quantitative determination of titanium dioxide (OLSMAN, 1969; LEE *et al.*, 1975). This method may be applied, however, only if the addition of titanium dioxide is compulsory to every soya product.

There are several methods described in the literature, to detect milk and/or soya proteins, based on polyacrylamide gel electrophoresis (FREIMUTH & KRAUSE, 1969; HOFMAN & PENNY, 1973; HOMAYOUNFAR, 1977; GUY & WILLCOX, 1977; VÁLAS-GELLEI, 1977; RICHARDSON, 1978). These methods are suitable only for qualitative analysis or, if SDS (sodium-dodecyl-sulfate) electrophoresis is applied, the evaluation of the method is more complicated because the number of fractions increases as compared to electrophoresis without SDS. The method of LEE and co-workers (1976) or HOFMAN (1977) is suitable for measuring quantities above 25% of non-meat proteins or describes indirect determination of them. Except for using Amido Black dye, the method of PARSONS and LAWRIE (1977) which describes thin-layer electrophoresis followed by laser-densitometric evaluation, seems to be reliable for quantitative purposes as well.

1. Materials and methods

1.1. Materials

1.1.1. Meat samples. For model experiments, deep-frozen pork and beef were used. Meat products containing either caseinate or soya protein were produced by the HUNGARIAN MEAT RESEARCH INSTITUTE and by the meat processing plants, in Budapest and Pápa.

1.1.2. Soya protein. The Promine D used was the product of the CENTRAL SOYA COMPANY (USA). Its protein content in the dry substance was at least

90%. Seventy-five % of the protein was water soluble. Promine D is a spray-dried product of 20–110 μ grain size.

1.1.3. Sodium caseinate. The employed sodium caseinate EM–HV was a product of the ZUID-NEDERLANDSCHE MELKINDUSTRIE N. V. (The Netherlands). Its protein content was more than 90%. At least 90% of the whole protein content was water soluble.

1.1.4. The chemicals used for preparing gel rods and buffer solutions were purchased from REANAL (Hungary). These chemicals are the following: acrylamide, N,N-methylenebisacrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), ammonium persulfate, glycine, urea.

1.1.5. Hydrochloric acid was a MERCK product.

1.1.6. Acetic acid was produced by ERDŐKÉMIA (Hungary).

1.1.7. Fast Green FCF dye was purchased from SERVA (Heidelberg).

1.1.8. The apparatus. To perform electrophoresis, PHARMACIA gel electrophoresis apparatus, type GE–4 and power supply unit, type EPS 500/400 were used. The apparatus functions with water-cooling and is suitable for the simultaneous insertion of 16 gel rods.

1.2. Methods

1.2.1. Treating and dissolving of samples

1.2.1.1. – Both sodium caseinate and Promine D standards were dissolved in 8 M l^{-1} urea. Concentrations applied were: 2 mg cm^{-3} in the case of sodium caseinate and 6.6 mg cm^{-3} in the case of Promine D.

1.2.1.2. Meat samples. – Fresh meat samples were minced, homogenized and apportioned. Thirty gramm portions were kept at 74 °C for 160 minutes in a drying oven, then, after cooling, deep-frozen and stored in the deep-freezer (–18 °C). The same heat treatment was applied both in the case of meat products of industrial origin and in the case of mixtures of meat–non-meat proteins prepared in our laboratory. Heat-treated industrial products were not subjected to heat treatment in the laboratory. Heat-treated products or samples formerly heat-treated and deep-frozen, were homogenized by mixing in a blender with 8 M urea solution. Thirty or 60 cm^3 quantities of urea solution were added to a 30 g sample. The solutions were filtered through cotton-wool or centrifuged. Filtrates or supernatants were used for electrophoresis.

1.2.2. Gel and buffer. Polyacrylamide gels and buffers were prepared according to ORNSTEIN (1964) and DAVIS (1964). The gels and buffer solutions were made to contain 8 M urea. Neither sample, nor spacer gels were used; only separation gel was applied. The protein samples were mixed with glycerol,

buffer and Bromophenol Blue and were layered on the gel surface. The acrylamide concentration was 9.4% and the BIS concentration 0.247%.

1.2.3. Electrophoresis. The upper reservoir was connected to the power supply as the cathode (origin), the lower reservoir as the anode (termination).

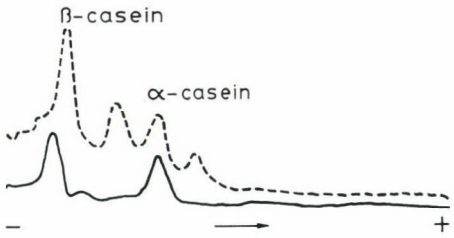

Fig. 1. Densitometric tracing of meat and sodium caseinate electrophoresis (——— sodium caseinate, – – – – meat containing sodium caseinate)

Electrophoresis was carried out with a current of 1.5–2.0 mA per tube and was continued until the front of the Bromophenol Blue reached a line about 5 mm from the lower end of the separation gel. The time requirement for electrophoresis was about 2.5 h.

1.2.4. Staining. The removed gel rods were immersed for 2 h in a 7% aqueous acetic acid solution containing 0.2% Fast Green FCF. Destaining was carried out by washing the gel rods in 7% aqueous acetic acid solution for about 72 h.

1.2.5. Evaluation of the experiment. Identification of the fractions was based on their relative mobility; quantitative determination was carried out by densitometric evaluation using *Chromoscan* MK II. (JOYCE LOEBL, England). Equations of calibration curves – to serve as basis to measure the unknown protein quantity – were determined from the standards according to the least

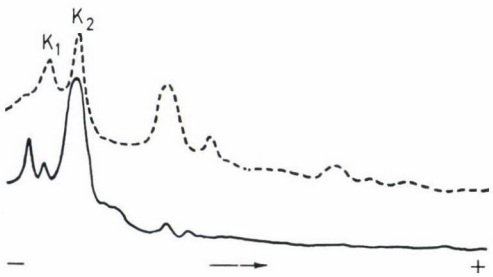

Fig. 2. Densitometric tracing of meat and Promine D electrophoresis (——— Promine D, – – – – meat containing Promine D)

squares method with the protein quantity and the areas of the peaks measured on the densitograms. The considered areas were in the case of milk protein those characteristic of the β-casein fraction and in the case of soya protein those characteristic of fractions K_1 or K_2 (Figs. 1–4).

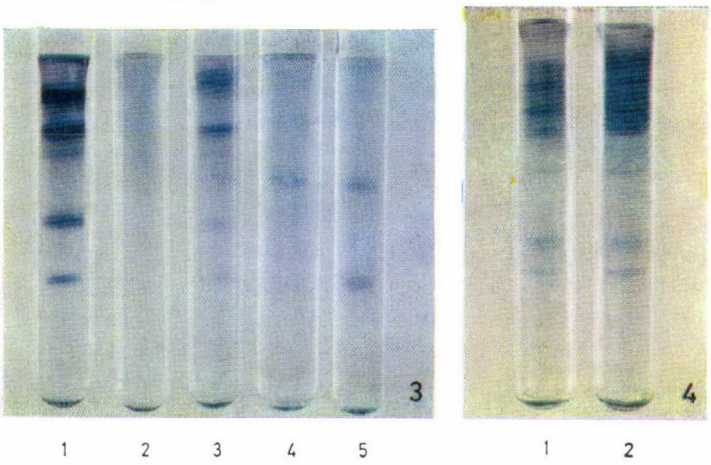

Fig. 3. Electrophoretic pattern of meat and sodium caseinate. 1: beef; 2: beef heat treated; 3: beef–sodium caseinate mixture; 4: beef–sodium caseinate mixture heat treated; 5: sodium caseinate

Fig. 4. Electrophoretic pattern of Promine D (1) and soya flour (2)

2. Results and discussion

2.1. Basic problems of quantitative determination

The quantitative determination of milk and soya proteins is based on the measurement of the intensity of their characteristic fractions separated by polyacrylamide gel electrophoresis. β-Casein ($R_M = 0.263 \pm 0.029$) was used to measure the quantity of milk protein whereas fractions K_1 and K_2 ($R_M = 0.106 \pm 0.031$ and 0.139 ± 0.033, resp.) were found to be useful for determining soya proteins. The method described in this paper was developed on the basis of that for electrophoretic separation and detection of soya and milk proteins in meat products (VÁLAS-GELLEI, 1977), however, quantification produced two additional problems. First a suitable dye had to be applied, the colour intensity of which is linearly proportionate to the quantity of the protein to be measured. Of the different dyes investigated (Amido Black 10B, Coomassie Brilliant Blue R–250 and Fast Green FCF), Fast Green seemed to be most convenient according to literary data (GOROVSKY *et al.*, 1970) and

proved to be most suitable in our experiments, too. Using the staining method as described in para. 1.2.4., dyeing is complete in the whole cross section of the gel rods and fractions do not become diffuse during destaining.

2.2. Effect of gel concentration on the qualitative results

The second question to be solved was the elimination of problems related to the denaturation of proteins (diffuse fractions, virtual decrease of quantity).

When examining aqueous solutions of milk proteins and urea solutions of soya proteins on gels of 7% acrylamide concentration in a basic system, we found good positive correlation with low standard deviation values between the protein concentration and the read off intensity values of the densitograms ($r = 0.982$–0.998, variation coefficient around $\pm 10\%$).

Protein fractions became diffuse as compared to those measured from pure solutions when the same proteins were mixed into meat. This fact greatly hindered quantitative evaluation and increased the standard deviation ($\pm 20\%$–$\pm 30\%$). To eliminate this disturbing effect, the gel concentration was increased without changing the proportion of acrylamide : bisacrylamide. Separating meat-sodium caseinate mixtures – treated previously according to para. 1.2.1.2. – and using gels according to para. 1.2.2., correlation coefficients of 0.981–0.999 were observed and the variation coefficient was less than $\pm 10\%$ (Table 1, Fig. 5). Separating protein mixtures of meat and

Table 1

Characteristic data of calibration curves in the quantitative determination of sodium caseinate

No.*	a	b	r	$\pm S_{xy}$ (%)
1	58.28	23.31	0.993	4.94
2	46.14	26.07	0.997	3.10
3	43.17	18.23	0.999	1.23
4	41.79	13.36	0.999	0.61
5	42.86	31.86	0.993	4.56
6	40.67	25.93	0.987	4.80
7	58.34	39.91	0.981	6.50
8	79.57	9.02	0.994	5.23
9	67.94	17.15	0.988	6.59
10	53.25	22.54	0.991	4.69
11	34.13	28.68	0.998	1.86
12	32.56	43.67	0.982	3.08

* Serial number of the different calibration curves

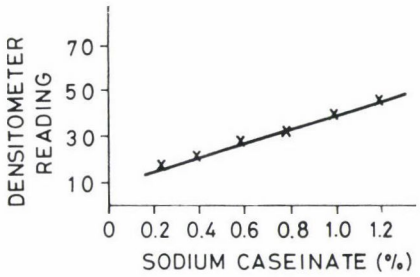

Fig. 5. Calibration curve of sodium caseinate determination. Densitometer reading means the integrated area of the characteristic protein fraction on the densitograms

soya in similar circumstances, correlation coefficients of 0.917–0.999 were found and the variation coefficient was less than $\pm 15\%$ (Table 2, Fig. 6). Addition of urea to a final concentration of 8 *M* resulted in quantitative dissolution of proteins.

The above mentioned modifications guaranteed not only good dissolution of the proteins but also their separation into sharp, distinct fractions and the possibility of quantitative dyeing.

Table 2

Characteristic data of calibration curves in the quantitative determination of Promine D

No.*	*a*	*b*	*r*	$\pm S_{xy}$ (%)
1	22.75	22.17	0.982	4.22
2	31.61	14.98	0.972	7.90
3	54.12	12.50	0.994	4.36
4	30.08	40.24	0.993	3.31
5	53.57	18.83	0.976	8.20
6	36.42	21.66	0.999	1.36
7	59.58	1.93	0.998	3.35
8	44.76	16.59	0.993	4.38
9	58.13	13.86	0.991	5.22
10	81.43	17.22	0.966	11.01
11	41.70	20.48	0.914	13.18
12	38.89	5.78	0.998	3.91
13	23.89	11.62	0.917	14.88

* Serial number of the different calibration curves

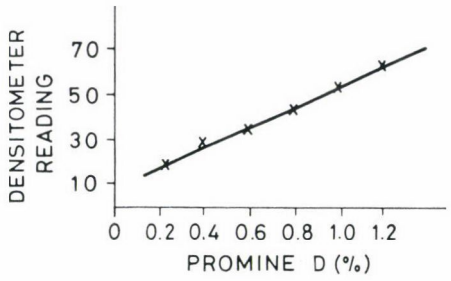

Fig. 6. Calibration curve of Promine D determination. Densitometer reading means the integrated area of the characteristic protein fraction on the densitograms

2.3. *Elimination of the disturbing effect of heat codenaturation of meat-milk proteins and meat-soya proteins*

These rather favourable results, however, were obtained in laboratory samples. In the case of industrial samples we got values lower than the declared sodium caseinate or Promine D concentrations, both when compared with pure protein or with mixtures of protein and meat (heat treated) as standards (in the former case the difference being 50%, in the latter 30–40%). Standard deviation was not higher than before.

The deviation observed in comparison to pure protein control may be explained by assuming codenaturation of meat and soya proteins during heat treatment in the course of the manufacturing technology (KOTULA & ROUGH, 1977). This phenomenon is presumed to apply to sodium caseinate, too, but in the case of heat-treated meat sodium caseinate, meat Promine D controls, error of such type was not to be expected. The degree of heat treatment of the standard samples was nearly the same as that of the industrial samples. In our model experiments alterations of either the duration or the temperature of the treatment did not cause any deviation that could be compared with the above-mentioned percentile error – within the range investigated. The considerable and consistently unidirectional error, observed in the determination of milk and soya proteins could not be explained by the uncertainty of the industrial sampling either. Thus – excluding other possibilities – it seemed possible that during heat treatment of the samples (see para. 1.2.1.2) codenaturation of meat protein–soya protein or meat protein–caseinate mixtures does not take place to the same degree as in industrial meat products. Since heat treatment was the same, deviation could occur only during storage. It was supposed that the process of codenaturation did not end with the termination of the heat treatment. In the subsequent 24–30 h, after cooling and during storage at —18 °C the intensity of soya and milk protein fractions

further declined. Thereafter samples could be stored deep-frozen practically without any change for any time (several months). The following standards were used for the determination: series of samples containing sodium caseinate or Promine D were prepared by mixing the proteins to be measured with 30 g of minced meat each, these were heat-treated in a thermostat at 74 °C for 150 minutes (in order to prevent loss of water, the samples were wrapped in aluminium foil); after cooling the samples were refrigerated and stored for at least 48 h in the deep-freezer. When put to use, standards were thawed and dissolved according to para. 1.2.1.2.

The application of a heat-treated standard is reliable. The fluctuation of the duration of heat treatment between 75 and 300 minutes or that of the temperatures between 60 °C and 90 °C do not result any significant difference. Storage from 48 h till 2 months does not cause any changes. Temperature or time values other than those mentioned, were not investigated.

2.4. Measuring range and standard deviation of the determination

Because of the possibility of long-time storage a number of standard samples may be prepared and stored simultaneously, thus at the arrival of samples, measuring may be started at once. Standards have to be applied to each electrophoresis. To obtain accurate results, it is important to perform both electrophoresis, staining and destaining under identical conditions. This may be done by destaining all the gel rods in the same destaining liquid (gel rods are put one by one into perforated glass tubes and placed in a beaker filled with destaining liquid). Under the above-mentioned conditions, when determining sodium caseinate or Promine D – dissolved from meat – a linear relation was found (within $\pm 15\%$ standard deviation) between protein concentration and densitometrically measured values (Tables 3–6). Linear relationship between protein concentration and the integrated area of densitometric peaks was found in the range of 24–80 μg soya or milk protein per gel rod. Thus the quantitative determination of 0.4% or more milk or soya protein in meat products becomes possible.

Non-meat proteins are expressed as percentage sodium caseinate and/or Promine D but other types of standards may also be used, or – in the knowledge of the type of non-meat protein – values may be converted by the use of adequate factors.

Table 3

Determination of sodium caseinate content in sausage "Párizsi" of a declared value of 0.5%

No.*	n	$\bar{x}$	s	V
1	4	0.44	0.04	9.1
2	4	0.65	0.04	5.4
3	4	0.46	0.02	4.6
4	4	0.68	0.05	6.9
5	4	0.58	0.04	6.9
6	4	0.60	0	0
7	4	0.49	0.02	4.1
8	4	0.49	0.02	4.1
9	4	0.47	0.03	4.3
10	4	0.46	0.01	2.2
1–10	40	0.532	0.089	16.7

* Serial number of the different examinations
n = number of parallel determinations
$\bar{x}$ = mean value
s = standard deviation
V = variation coefficient (100 $s/\bar{x}$)

Table 4

Determination of sodium caseinate content in sausage "Párizsi" of a declared value of 2.0%

No.*	n	$\bar{x}$	s	V
1	5	1.80	0.10	5.6
2	4	2.14	0.10	4.7
3	4	1.88	0.07	3.7
4	4	2.40	0.11	4.6
5	4	1.67	0	0
6	4	2.53	0.04	1.6
7	4	2.13	0.11	5.2
8	4	1.99	0.09	4.5
9	4	1.93	0.09	4.7
10	4	2.14	0.08	3.7
1–10	41	2.061	0.257	12.5

For legend see Table 3

Table 5

Determination of Promine D content in sausage "Párizsi" of a declared value of 0.5%

No.*	n	x	s	V
1	4	0.46	0.03	6.5
2	4	0.45	0.04	8.7
3	4	0.46	0.02	5.0
4	4	0.45	0.02	3.8
5	4	0.47	0.02	4.3
6	4	0.53	0.04	7.5
7	4	0.43	0.04	9.3
8	4	0.53	0.03	5.7
9	5	0.48	0.04	8.33
10	5	0.48	0.03	6.3
1–10	42	0.475	0.042	8.8

For legend see Table 3

Table 6

Determination of Promine D content in sausage "Párizsi" of a declared value of 2.0%

No.*	n	$\bar{x}$	s	V
1	4	2.00	0.12	6.1
2	4	1.91	0.06	3.1
3	4	1.94	0.04	1.8
4	4	1.86	0.06	3.2
5	4	2.14	0.08	3.7
6	4	2.15	0.14	6.5
7	4	1.97	0.11	5.6
8	5	1.93	0.08	4.1
9	5	2.06	0.07	3.4
10	4	2.02	0.05	2.3
1–10	42	1.995	0.123	0.3

For legend see Table 3

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OBJECTIVE METHODS FOR THE INDICATION OF POST-RIPENING AND OVER-RIPENING IN STORED APPLES

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Post-ripening of apples is a process of positive character since in its course the sensory properties improve. Post-ripening is, however, followed by over-ripening and this is unambiguously a process of senescence. During this period the sensory properties deteriorate and simultaneously storage diseases advance.

The quality index as suggested by THIAULT (1970) proved to be characteristic of the tendencies of post-ripening during storage [total sugar content (g l^{-1}) + 10 × titratable malic acid (g l^{-1})].

In apples stored with a higher storage potential this quality index increased during a longer period (Fig. 2) than in apples of lower storage potential. In controlled atmosphere apples stored with practically identical storage potential showed an extended positive post-ripening period (Fig. 3).

The continuous decrease in quality is an undesirable process because it shows not only a decline in quality but, in its course, it increases the proportion of apples attacked by storage diseases (Fig. 4, A, B, C).

Jonathan apples possess a high quality index. During storage in normal atmosphere this index declines at first at a lower rate but later at an increased rate. This phenomenon should be attributed unambiguously to senescence, since parallel to the decrease in the quality index increased the proportion of apples attacked by storage diseases.

The quality index as established by THIAULT (1970) is a summary of the respiration substrates, while the physiological importance of acid breakdown is revealed by multiplication by ten. Possibly this index, further developed and modified may become suitable for the control of the ripening intensity of stored apples.

Apples for storage should be picked most advantageously in the pre-climacteric stage of ripening, because, according to DILLEY (1965), this is the stage in which the storage potential is at its highest. The storage potential varies with apple variety as the reserve nutritives differ significantly at the time of the beginning of ripening (KÁLLAY, 1976, 1980). Ripening and thus post-ripening during storage as well is essentially a process of senescence (BIALE, 1960). The storage capacity of an apple variety of given storage potential depends on the conditions of storage. In relation to cold storage ripening processes are slower in controlled atmosphere or in vacuum chambers (DILLEY, 1977) where their intensity may be repressed to the approximate ideal conditions (HULME & RHODES, 1971).

With the aid of modern storage technology the ripening of apple may be slowed down to the extent enabling a detailed study of the process. Although ripening as a process of senescence is accompanied by the disorganization of

cell life (McGLASSON, 1970) which leads in the end to the deterioration of the apple, from the aspect of consumption it may not be considered a fully negative phenomenon, since the fruit becomes suitable for consumption just through ripening.

In storage practice it is very important to remove the apple from storage at the optimum of its sensory quality. If the apple becomes overripe during storage that means lower quality for the consumer and strongly increased storage loss for the grower.

The aim of this study was to find indices by which post-ripening may be distinguished from over-ripening satisfactorily.

1. Materials and methods

Among other varieties, the post-ripening of two apple varieties, that of the rapidly ripening *Jonathan* and of *Starking*, characterized by significantly lower ripening intensity, was studied under normal storage conditions and in controlled atmosphere. During the storage period, every month 10 apples were removed from the lots. These were first thoroughly inspected then tested in the laboratory. The consistency of the flesh was tested by a *Magness Taylor* penetrometer. The composition (total sugar, titratable acid) was analysed according to THIAULT (1970). To evaluate the quality changes in apple during storage the quality index as established by THIAULT (1970) was used [total sugar (g l^{-1}) + 10 × free acid (g l^{-1})]. THIAULT (1970) found a close correlation between this quality index and the scores given by sensory panels. Of the physiological disorders occurring during storage those relating to the senescence of individual tissues or of the total apple were given special attention (KÁLLAY & KEMENES, 1977). The loss upon storage was expressed by the proportion of apples attacked by diseases. Diseases accounted for were: lenticel spot, *Jonathan* spot, senescent-internal breakdown, wilting. Scald was not evaluated since it may be eliminated by treatment with chemicals.

2. Results

2.1. Changes in the consistency during storage

Figure 1 shows the behaviour of four apple varieties of different storage potential during storage in normal atmosphere.

Post-ripening may be considered concluded when the apple flesh firmness reaches the minimum level required in the trade. Consistency of flesh is a characteristic important enough to determine the fate of the stored

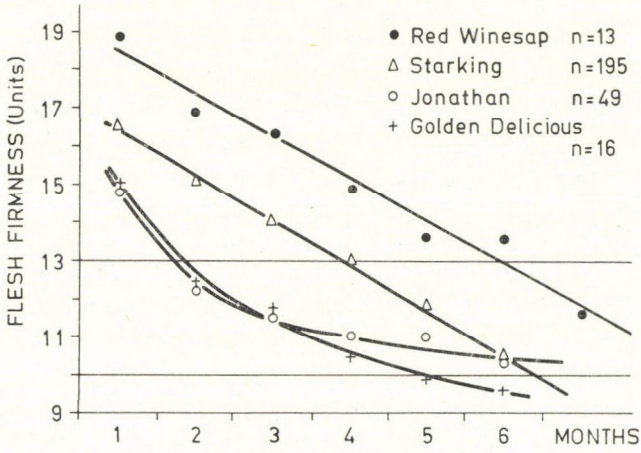

Fig. 1. Consistency of different apple varieties as measured with *Magness Taylor* penetrometer

apple. If the apple reaches a level of softness above a certain limit (Fig. 1, 13–10 Units) it must be considered over-ripe, regardless of other quality criteria. In varieties *Red Winesap* and *Starking* the end of post-ripening may be easily established, since the process of softening is a linear one. The softening in varieties *Golden Delicious* and *Jonathan* may be described by a flattening curve. Thus in these varieties the end of post-ripening may not be reliably characterized by measuring solely flesh firmness.

2.2. Changes in the quality index during storage

In Fig. 2, the quality indices of *Starking* apples, picked at different times and stored under normal atmosphere, are shown. The time of picking was set in relation to the day of full bloom. The samples (a total of 195) were taken from different orchards.

Starking apples contain significant amounts of reserve nutrients at the beginning of storage. These reserves, however, diminish as an effect of ripening on the tree and thus the quality index of apples picked later increases during a shorter period. It is characteristic that the quality index of apples picked on the 125th or 130th day did not decrease even after 6 months of storage, while that of those picked on the 160th day hardly increased at all. The high storage potential of this variety is shown by the fact, that except for the fruit picked at extremely late dates, the quality index was higher after 6 months storage than at the beginning of the storage period. In the formation of the quality index an optimum was observed, dependent on the storage potential at the beginning of storage and on the time of picking. In the case of a more advanced storage technology the quality improving phase is extended.

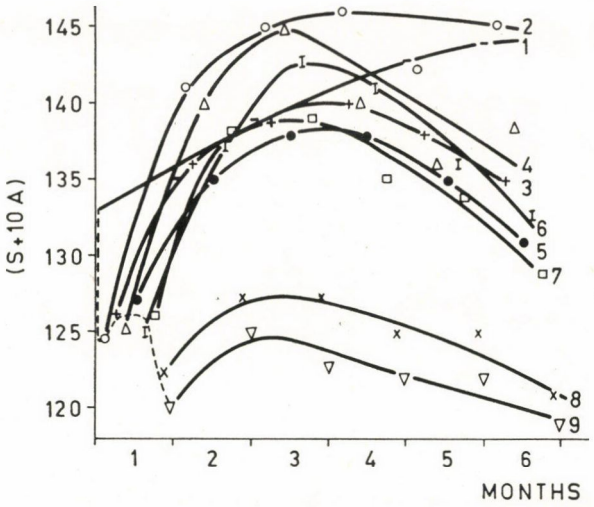

Fig. 2. Quality index of *Starking* apple picked at different times. Changes in the quality index (sugar + 10×acid content) on the tree: dashed line; during storage: solid line. 1. Picked on the 125th day; 2. on the 130th day; 3. on the 135th day; 4. on the 140th day; 5. on the 145th day; 6. on the 150th day; 7. on the 155th day; 8. on the 160th day; 9. on the 165th day after full bloom

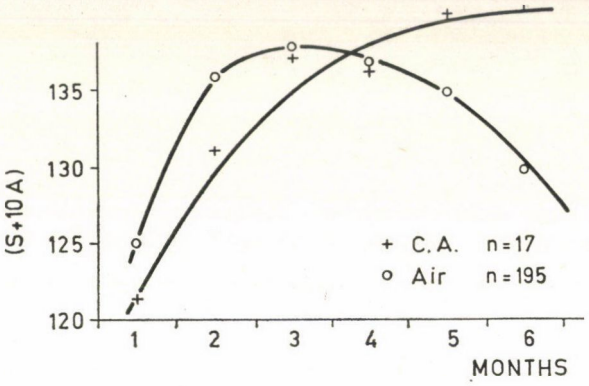

Fig. 3. Quality index of *Starking* apples during storage in normal and controlled atmosphere

Storage in controlled atmosphere facilitates an extended post-ripening of apples of a given storage potential.

The average values of the 195 samples stored under normal conditions were compared with those stored in controlled atmosphere. The latter comprised 17 samples picked between the 140th and 160th day. The optimum of post-ripening was extended by several months in the atmosphere containing 3% CO_2 and 4–5% O_2.

The question is whether the end of post-ripening coincides with the development of the maximal quality index. If this was true, the beginning of over-ripening could be marked by the decrease of the quality index. Since, however, over-ripening is an aging process, it seemed necessary to follow up also the frequency of storage diseases in *Starking* apples. Storage diseases hinting at over-ripening were found, however, in insignificant number (under 1%).

During the relatively short storage period (6 months) of the *Starking* apples the positive period of post-ripening could be observed: in the first 3–4 months of storage the apples were characterized by good consistency and

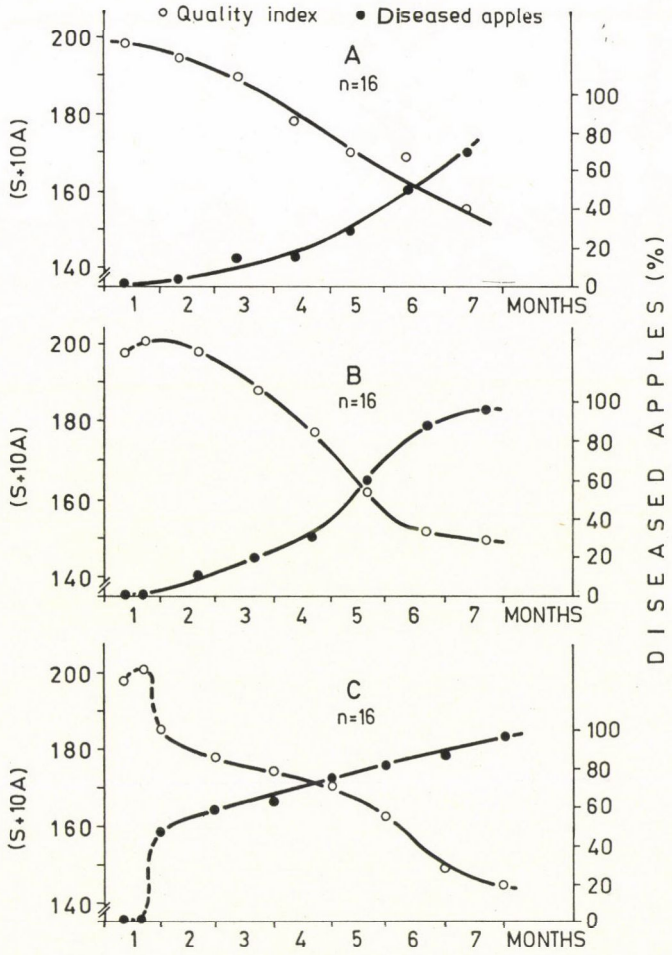

Fig. 4. Changes in the quality index and proportion of diseased *Jonathan* apples during storage. A: picked on the 130th day; B: on the 140th day; C: on the 150th day; dashed line: changes on the tree; solid line: during storage. ○ = quality index; ● = diseased apples

optimal quality index. The symptoms of over-ripening could not be sufficiently studied because in the 5–6th months of storage only initial signs became apparent.

Further formation of the quality index could be followed up during the storage of *Jonathan* apples of a much lower storage potential. Under normal storage conditions the quality index of *Jonathan* apples showed a diminishing tendency (Fig. 4). The decrease of the quality index and the storage diseases due to senescence showed a close correlation and both variables could be explained by the difference in the storage potential.

The *Jonathan* apples as seen in Fig. 4, were picked from a split-plot system experimental area. The experimental results were obtained during 1976 for which season a dry summer and abundant rain during the harvest was characteristic. These conditions accelerated ripening or aging on the trees (Fig. 4, C). The experiment was repeated during the subsequent years (1977, 1978, 1979) under similar conditions. Although the effect of the different years is apparent, the results, apart from the year 1979, apples of the 130th day show again close correlation between the two characteristics of senescence: reduction of the quality index and increasing proportion of diseased apples.

Table 1

Quality index of aging Jonathan apples and the proportion of diseased apples in different experimental years

Year	Day of picking related to full bloom days	Quality index at the beginning of storage period	Quality index at the end of storage period	Ratio of diseased apples at the end of 7 months	Correlation of quality index and proportion of diseased apples
1976	130	198	155	70	$r = 0.95$
	140	202	151	97	$r = 0.98$
	150 (202)	185	145	97	$r = 0.95$
1977	140	193	165	22	$r = 0.85$
	150 (193)	182	151	48	$r = 0.89$
1978	130	204	169	59	$r = 0.90$
	140 (204)	199	165	59	$r = 0.89$
	150 (204)	200	158	93	$r = 0.94$
1979	130	198	180	30	$r = 0.45$
	140	207	170	70	$r = 0.84$
	150 (207)	206	160	84	$r = 0.95$

Note: Quality indices in parentheses are the peak values during ripening and were used as the basis of comparison in further calculations

In the case of the exception the reduction in the quality index was preceded by slight appearance of lenticel spots. The latter did not increase in the course of four months, however, it spoiled the correlation.

3. Conclusions

In apples of high storage potential, *i.e.* of high water-insoluble reserve carbohydrate content, the post-ripening during storage is a positive process and the quality index of the fruit shows an improving tendency, though to an extent influenced by the storage technology applied. This advantageous change occurs when the rate of diminishing of the respiration substrates dissolved in the cell sap (sugars and acids) is exceeded by that of the hydrolysis of the reserve carbohydrates. No change in the quality index may infer a dynamic equilibrium which is possible if, simultaneously with a continuous, though not constant rate of breakdown of acids, the sugar content of the cell sap sufficiently increases. Reduction of the quality index may be caused by a shift in the dynamic equilibrium inferring a relative predominance of respiratory activity. Although it is generally accepted that the biological foundation of fruit storage is invested in the respiration characteristics, for practical reasons it seems more advisable to measure the concentrations of respiratory substrates, than the respiration intensity very difficult to measure under storage conditions (respirometers). According to the observations of the author, post-ripening continues till the quality index is on the rise. Over-ripening begins with the decline of the quality index, and this is independent of the circumstances that the highest quality index is reached during ripening on the tree or during the slower ripening in storage. The worst phase of over-ripening – in which storage diseases may occur – apparently begins when the quality index becomes lower than at the start of storage.

In *Jonathan* apples, characterized by rapid ripening, the major part of ripening takes place on the tree. The extent of reduction as compared to the highest value of the quality index (Table 1) may not exceed 25–30%. The rate of decrease in the quality index is not uniform, there exists always a temporary accelerated phase before and after which the rate of change is more moderate. The dynamics of storage diseases runs parallel with the change in the quality index. This shows that disorganization of cell life becomes dominant in this transitory phase, which is manifested by the appearance of spots and leads finally to the total breakdown of the fruit. The reduction of the respiration substrates is slight in the phase preceding total spoilage. Respiration in disorganized cells becomes minimal, in the reduction of the respiration substrates, oxidative processes become dominant (browning or drying out of the flesh).

In storage practice, the measurement of consistency and the change in the quality index present very useful information. These two objective criteria, although over-ripening as indicated by them may not always coincide, have an important complementary value. The highest quality index signifies the peak of ripening from the aspect of the consumer. The extent of reduction following the peak seems to be in relation with the proportion of diseased apples, in particular with *Jonathan* apples.

Correlation between the reduction in the quality index (%) and the proportion of diseased apples in the average of four years of observations can be demonstrated below:

Reduction of the quality index (%)	0	5	10	15	20	25	30
Diseased apple (%)	1.4	17.4	33.3	49.4	65.2	81.2	97.1
Deviations around the mean (±)	5.5	4.0	3.4	4.1	5.6	7.4	9.4

No such correlation can be found between the deterioration of consistency and the dynamics of apple diseases, since softening of the flesh is a consequence of the solubilization of pectic substances and the latter may be considered, to a certain extent, as reserve carbohydrates.

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A STUDY OF POSSIBLE MUTAGENICITY OF IRRADIATED ONION POWDER BY SALMONELLA/MAMMALIAN-MICROSOME TESTS

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The Salmonella/mammalian-microsome mutagenicity test was applied to detect possible mutagenicity of onion powder irradiated with 5 and 10 kGy, resp. Aqueous extracts and pepsin/pancreatin digests equivalent to 50 and 20 mg of onion powder per plate, resp., were tested with and without the rat liver microsomal fraction, with *Salmonella typhimurium* mutant strains TA 100, TA 1535, TA 98, TA 1537 and TA 1538. Within the limitations of the experimental conditions applied, no mutagenicity of irradiated onion powder was demonstrable.

Technological feasibility of radiation decontamination of dehydrated onions has been thoroughly studied and its dose requirement was established (FARKAS & EL-NAWAWI, 1973; SILBERSTEIN *et al.*, 1979a, b). Chemical changes of onion powder proved to be minute and practically insignificant after doses necessary to sufficient reduction of the viable cell count or even to sterilization (GALETTO *et al.*, 1979).

In the frame-work of extensive studies on the wholesomeness of irradiated spices and seasonings, performed in Hungary as a contribution in kind to the International Project in the Field of Food Irradiation (IFIP, Karlsruhe, FRG), mutagenicity testing of irradiated ground paprika, black pepper and a spice mixture, resp., has already been reported (ANON., 1977; FARKAS *et al.*, 1981). As a part of this programme, the Salmonella/mammalian-microsome mutagenicity test was applied to detect possible mutagenic activity of irradiated onion powder.

1. Materials and methods

Commercially produced onion powder was irradiated with 5.0 and 10.0 kGy doses under aerobic conditions by a ^{60}Co gamma radiation source at ambient temperature (dose rate: 7.0 kGy · h^{-1}).

After 2–4 weeks of post-irradiation storage at 4 °C, samples of untreated and irradiated onion powder were extracted with distilled water (onion powder, 1 part + water, 9 parts) in a shaker bath at room temperature for 60 minutes, then they were centrifuged and the supernatants were filter-sterilized by membrane filtration (pore size of the filter: 0.2 μm).

After 6–8 weeks of post-irradiated storage of the samples of onion powder, enzymatic digests (PHILLIPS & ELIAS, 1978) were also prepared to simulate mammalian digestion previous to mutagenicity test as follows: 3 g of onion powder were suspended in 27 ml dist. water and the pH value of the suspension was adjusted to pH 2.0 with 10 *N* HCl. 15 mg of crystallized pepsin (*Merck*, 100 mU mg^{-1}, Cat. No. 7192) were added and the mixture was incubated in a thermostated shaker bath at 37 °C for 3 hours. After this incubation its pH value was raised to pH 7.5 by 5 *N* NaOH, then 300 mg pancreatin (*Merck*, Cat. No. 7130) and 15 mg sodium taurocholate were added, and the incubation was continued for another 6 hours. The digest obtained was separated from the undigested material by centrifugation and the supernatant was mixed with an equal volume of Me_2SO. After filter-separation of the pectic precipitate formed, the clear digest was sterilized by membrane filtration.

Table 1

Reversion test with aqueous

Sample added	Number of revertants			
	TA 100		TA 1535	
	−S9	+S9	−S9	+S9
0 kGy	134 ± 28	143 ± 11	32 ± 5	15 ± 4
5 kGy	124 ± 18	134 ± 10	34 ± 11	16 ± 1
10 kGy	173 ± 38	133 ± 10	35 ± 12	31 ± 2
None (spontaneous reversion)	109 ± 11	129 ± 9	25 ± 1	15 ± 3
NaN_3, 1 µg per plate	972 ± 143	867 ± 130	912 ± 8	300 ± 37

* Extracts were prepared after 2—4 weeks of post-irradiation storage at 4 °C.

Table 2

Reversion test with enzymatic

Sample added	Number of revertants			
	TA 100		TA 1535	
	−S9	+S9	−S9	+S9
0 kGy	135 ± 5	131 ± 7	42 ± 2	19 ± 2
5 kGy	130 ± 5	144 ± 13	31 ± 3	15 ± 2
10 kGy	135 ± 6	142 ± 7	41 ± 5	13 ± 1
None (spontaneous reversion)	121 ± 2	126 ± 5	22 ± 1	11 ± 1
Aflatoxin B_1, 0.1 µg per plate	—	382 ± 25	—	17 ± 4
NaN_3, 1 µg per plate	1519 ± 140	1118 ± 36	1187 ± 330	289 ± 75

* Enzymatic digests were prepared after 6—8 weeks of post-irradiation storage at 4 °C.

Half cm^3 aliquots of aqueous extracts and 0.4 cm^3 of enzymatic digests, resp., were tested for mutagenicity, with and without the liver microsomal fraction from albino rats induced by *Aroclor* 1254, with *Salmonella typhimurium* mutant strains TA 100, TA 1535 (base-pair mutants), TA 98, TA 1537 and TA 1538 (frameshift mutants) as described by AMES and his coworkers (1975). The S9-mix added per plate contained 50μl S9. The mutability of the strains and their genotypes were checked according to recommended procedures. Sodium azide and aflatoxin B_1 were used as positive controls. After incubation of the plates in the dark for 48 h at 37 °C, revertant his^+ colonies were counted. Application of considerably higher concentrations of the onion extracts seemed to us to be impractical taking into account their histidine content and/or possible toxicity (GALETTO *et al.*, 1979; SILBERSTEIN *et al.*, 1979b).

*extracts of onion powder**

(mean ± S.D. of triplicates)

TA 98		TA 1537		TA 1538	
−S9	+S9	−S9	+S9	−S9	+S9
66 ± 5	88 ± 32	16 ± 4	35 ± 13	37 ± 5	21 ± 2
62 ± 10	112 ± 6	10 ± 3	26 ± 3	27 ± 8	23 ± 3
72 ± 15	99 ± 47	11 ± 6	35 ± 8	27 ± 3	24 ± 8
52 ± 5	61 ± 4	10 ± 1	8 ± 3	28 ± 2	30 ± 2
51 ± 6	62 ± 12	10 ± 2	7 ± 1	31 ± 2	35 ± 3

*The amount of aqueous extract applied was equivalent to 50 mg of onion powder per plate

*digests of onion powder**

(mean ± S.D. of triplicates)

TA 98		TA 1537		TA 1538	
−S9	+S9	−S9	+S9	−S9	+S9
76 ± 6	83 ± 5	18 ± 3	19 ± 3	29 ± 6	38 ± 3
61 ± 3	91 ± 9	11 ± 2	24 ± 3	27 ± 1	32 ± 3
76 ± 8	77 ± 5	21 ± 7	20 ± 3	33 ± 5	34 ± 5
38 ± 5	63 ± 6	7 ± 1	14 ± 5	28 ± 1	31 ± 3
—	1395 ± 88	—	37 ± 4	—	153 ± 6
43 ± 3	65 ± 7	8 ± 1	9 ± 1	28 ± 1	35 ± 3

*The amount of enzymatic digest applied was equivalent to 20 mg onion powder per plate

2. Results and discussion

Results of reversion tests are shown in Tables 1 and 2. The applied amounts of the aqueous extracts and enzymatic digests were equivalent to 50 and 20 mg of onion powder per plate, resp.

No significant increase in the number of revertants was found with groups of onion powder samples irradiated with 5 and 10 kGy as compared with the unirradiated controls. For all reported data the revertant colonies obtained were typical and the light "background lawn" was normal in appearance indicating that non-toxic amounts of the extracts were tested. Plate counts made after incubation of tester strains in the aqueous extracts of onion powder for 1 hour at 37 °C showed no bactericidal effects (Table 3).

Table 3

Plate counts of tester strains before (B) and after (A) incubation for 60 min at 37 °C in aqueous extracts of onion powder

Onion extract	TA 100		TA 1535		TA 98		TA 1537		TA 1538	
	B	A	B	A	B	A	B	A	B	A
0 kGy	55*	43	250	256	117	187	37	21	81	190
5 kGy	59	43	247	244	123	187	36	23	79	196
10 kGy	53	46	244	248	120	202	34	22	77	198
Control (without onion extract)	55	64	247	240	123	122	37	40	64	85

* Mean of triplicates

The strains detecting base-pair mutations (TA 100 and TA 1535) showed a strong mutagenic response to NaN_3 applied at the 1 μg per plate level. Our observations on the mutagenicity of sodium azide with and without S9-mix confirm the findings of DE FLORA and co-workers (1979) that azide mutagenicity can be decreased in the presence of liver post-mitochondrial fractions. One-tenth μg aflatoxin B_1 per plate induced a significant increase of reversions in tester strains except in TA 1535.

Within limitation of experimental conditions applied, and in accordance with various mutagenicity studies on low-dose irradiated onions (SHILLINGER, 1973; ZAJTSEV *et al.*, 1974; ANON., 1976; MOHYUDDIN, 1976; HATTORI *et al.*, 1979), the conclusion is drawn that no mutagenic activity of high-dose irradiated onion powder was demonstrable by the Salmonella/mammalian-microsome mutagenicity tests.

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THE EFFECT OF PROCESSING TECHNOLOGIES ON COLOUR CHANGES IN TOMATO

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Data on colour development during tomato processing are reported. Samples were taken on 3 occasions during 24 h representing different processing phases at 6 different points of the processing line. The 7th sample included a paste stored for 2 months. Samples were taken at 30-min intervals and measured in a *Hunter* D25–D3 colorimeter.

The relatively high standard deviations of pulp colour overlap several quality classes and diminish gradually to one class in the course of processing. It appeared again that colour deteriorates to a higher degree in raw material with good colour than in that with poorer colour. The highest colour deterioration was found in the pulp and the heated and strained juice with ΔE_{Lab} values of 2.20. Between the raw material delivered and the finished paste an average reduction of 0.47 *a/b*, $\Delta E_{Lab} = 4.35$ was measured, which corresponds to nearly two and a half colour class categories.

On the processing line in question, 0.40–0.50 *a/b* colour reduction can be reckoned with. Accordingly, raw material of at least 2.5 *a/b* value is needed to produce pastes of $\Delta 2.0$ *a/b*. The author concludes that reduction in heat treatment during the different processing phases might improve colour.

On a pilot tomato processing line, samples were taken on 3 occasions during processing in order to verify changes in tomato colour.

Information on colour changes during processing was required from instrumental analysis of samples taken regularly at 7 points of the processing line during 24 h.

Samples were taken from:

1. Pulp collecting tanks. There were 2 types of these. One for pulp obtained in field juice stations; the other inserted in the line after the pulper and seed separator.
2. Tank of the heated, strained tomato juice.
3. Juice tank in front of the evaporator.
4. Evaporated paste, puree of 28–30% refr. index obtained from the vacuum evaporator.
5. Paste heated and filled into tins immediately prior to sealing.
6. Paste sterilized and cooled as the tin size required.
7. Goods stored uniformly for 2 months at 20 °C.

On the line temperature and pressure conditions were identical. An old type of P15 equipment was used with a *Zsigmond* type pre-heater.

Filling varied highly. At the time of sampling 5/1-, 1/1- and 1/2-kg tins and 3/4-kg jars were filled with a paste of 28–30%; then during 12 h, salted products of a total solids content of 38–40% were filled in barrels. In the next 12 h 15-kg tins were produced.

In the 3 trial periods, different cooling systems were used. In the first study, continuous cooling was applied in a tunnel; in the second, cooling water was renewed after warming up; in the third, *Minusol* cooling liquid was used.

1. Materials and methods

Tomato pulp of commercial quality and whole fruits were processed. Samples taken at the different sampling points at 30-min intervals were brought in 1 kg boxes to the laboratory.

Pulp was passed through a *KÖVAC* IKP–006 pulping machine with 0.8 mm mesh sieve and de-aerated by vacuum.

Samples of strained juice and paste were cooled to about 20 °C with tap water prior to measuring.

Prior to measuring, paste was diluted to 9.0 $\pm$0.1% solids with distilled water according to international standard.

Colour was measured in *Hunterlab* D25–D3 digital colour difference meter.

Diffuse, wholly concentric illumination was applied with 45°–0° geometry. The measurement surface had a diameter of 102 mm illuminated with a quarz-halogen lamp of *cca.* 350 lumen intensity. The 4 silica photodiode sensors were equipped with 4 broad-band filters.

For measurements, plastic cuvettes of 110 mm inside diameter and 60 mm height covered by optical glass were used. They were filled up to 40 mm and covered with black cover.

The L, a, b values of the *Hunter* colour system have the advantage that the a and b values, resp., characterizing colour, coincide with the red–yellow ratio indicating the colour or redness of tomatoes. Thus their index was chosen as colour standard for tomato pastes in several countries expressed as the a/b ratio.

In evaluating colour, a/b values of 2.0 and 1.8 mostly adapted in international practice were used.

Accordingly:

- Class I tomato pastes, juice, sauce, *etc.*, have a/b values of 2.0 or above.
- Class II tomato pastes have 1.8–1.99 a/b ratio.
- Class III products have 1.60–1.79 a/b values.

Moreover, as 0.2 a/b ratio equals one class interval, samples

- between 2.2–2.4 a/b ratio were classed as extra
- between 2.4–2.6 a/b ratio as super extra.

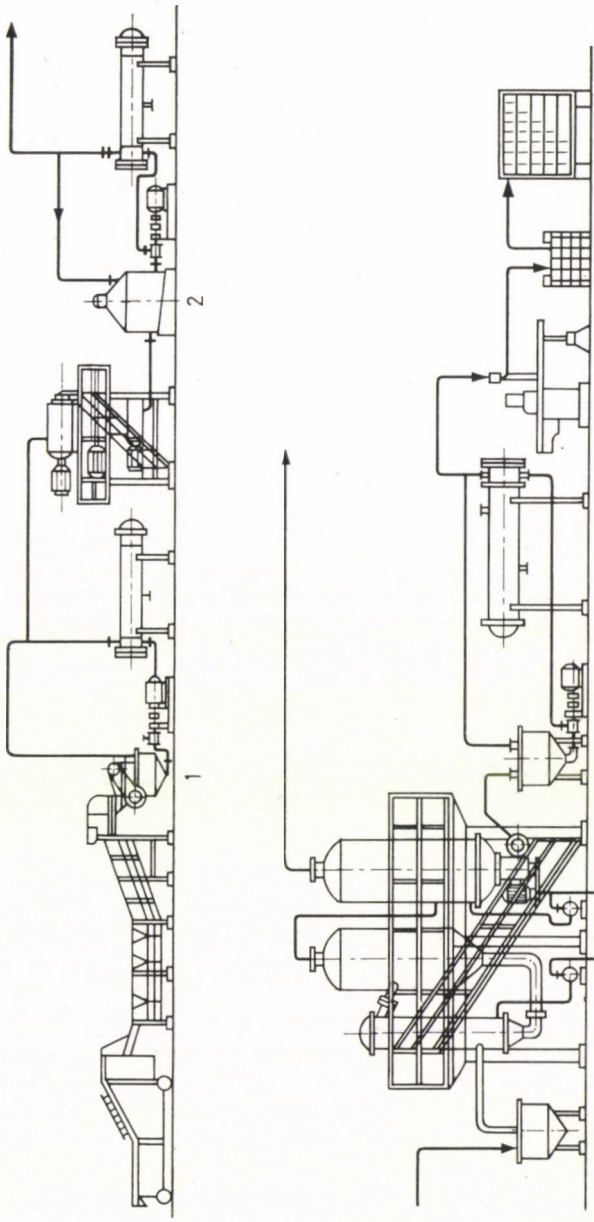

Fig. 1. Pilot processing line with sampling points: 1. pulp tank; 2. strained juice tank; 3. juice tank in front of evaporator; 4. paste tank after evaporation; 5. paste filled in tins; 6. sterilized paste; 7. paste stored for 2 months

Two index values can be used to measure colour differences. One of them indicates actual differences between a/b ratios, the other calculates colour differences using the ΔE_{Lab} formula introduced by CIE:

$$\Delta E_{Lab} = (L^2 + a^2 + b^2)^{1/2}$$

$E_{Lab} = 1.0$ value is the threshold value for the discerning capacity of the human eye. No colour differences below 1.0 can be perceived by the eye.

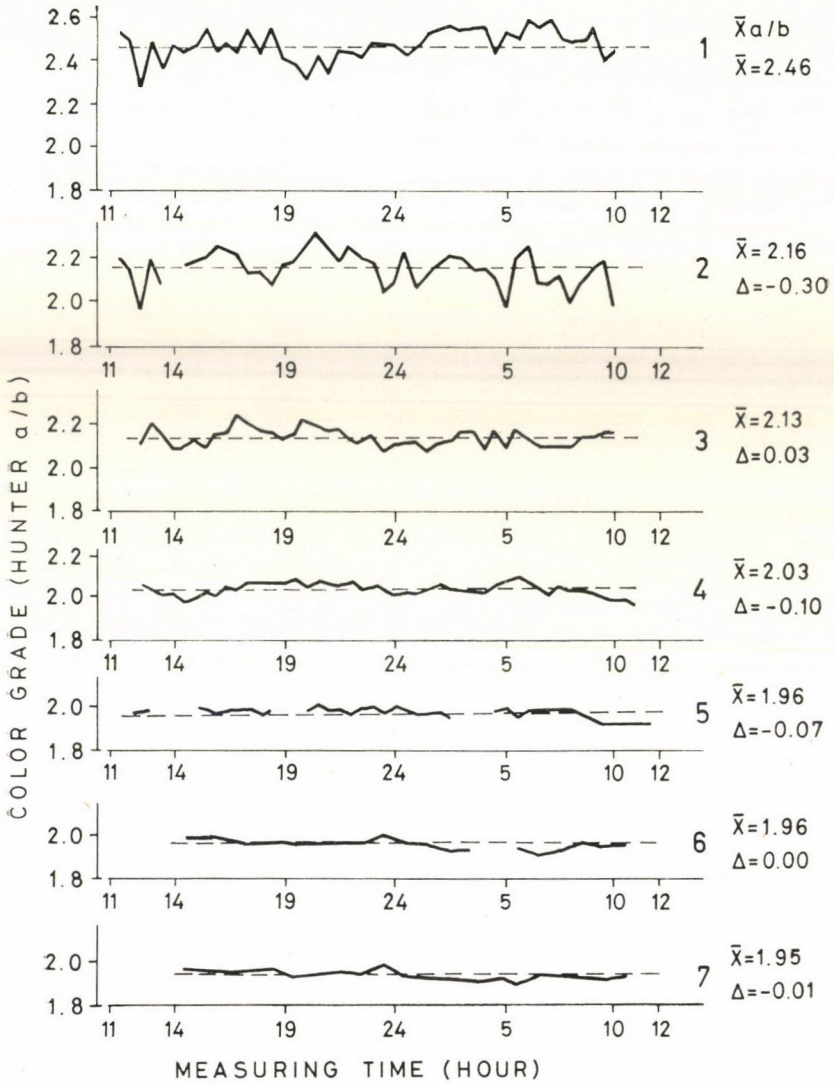

Fig. 2. Colour development at different sampling points during processing. Results of measurement 1

2. Results

Salient changes (whether increase or decrease) in the pulp and strained juice serving as the raw material of the samples appear relatively isolated even in the later phases of technology. Depending on progress and time of processing, its curve will level out and shift in time.

The least coincidence was found in the pulp–strained juice set.

Inhomogeneity of the brought-in raw material colour was indicated by high "deviation values".

Figure 3 represents average colour development in the raw material and finished products of the 3 measurement series and minimum and maximum value changes at the different sampling sites.

In the upper left corner, mean colour values of the pulp measured in 24 h are shown; in the lower right corner, colour values of the sterilized, finished product according to mean data obtained after 2 months of storage.

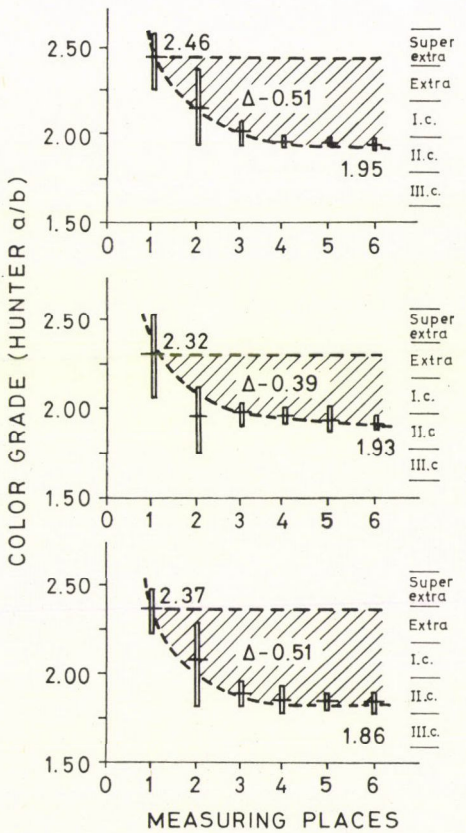

Fig. 3. Effect of processing operations on colour quality development at different sampling points

The shaded part represents the imaginary area limited by colour development of the raw material and finished product.

The number marked with delta in the middle of the shaded area indicates the difference between the mean colour value of the pulp and that of its paste.

Figure 4 represents the totalled mean values of the 3 processing lines.

In the 3 measurements, raw material of an average 2.38 a/b colour value yielded 1.91 a/b ratio products.

The difference between the two values (0.47 a/b) corresponds to nearly two and a half colour categories.

Extreme values and their level at the different sampling points are also given.

Figure 5 represents the density function of data measured at the sampling points.

It sums up the colour development and concentration in the pulp during processing.

It appears that pulp colour values scatter over several quality categories while those of the paste are concentrated on a much closer area including only one category.

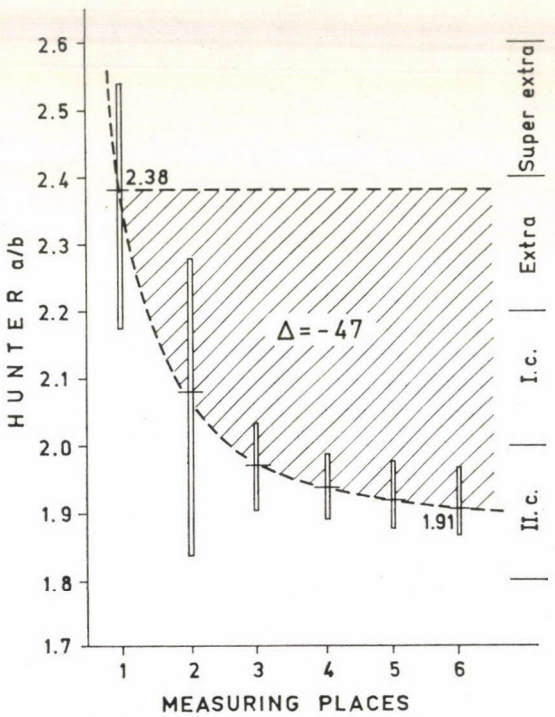

Fig. 4. Extreme and mean values of samples in the average of 3 measurements, according to a/b ratio

Former studies revealed more considerable colour deterioration in raw material of good colour than in raw material of poorer colour quality. Can this fact alone explain the considerable change in colour we experienced?

In the first measurement, the mixture of 80% super extra (2.4–2.5 *a/b*) and 20% extra (2.2–2.4 *a/b*) quality pulp yielded 36.6% extra and 57.8% class I strained juices. The serious colour reduction indicated that preparation took too long a time.

Juice colour in the tank in front of the evaporator differed significantly from that of the strained juice indicating renewed heat effect. The colour of the 2 samples ought to have been identical.

Tomato puree obtained from the evaporator included 89% class I and 11% class II products.

Heating prior to filling resulted in 2.6% class I and 97.4% class II products.

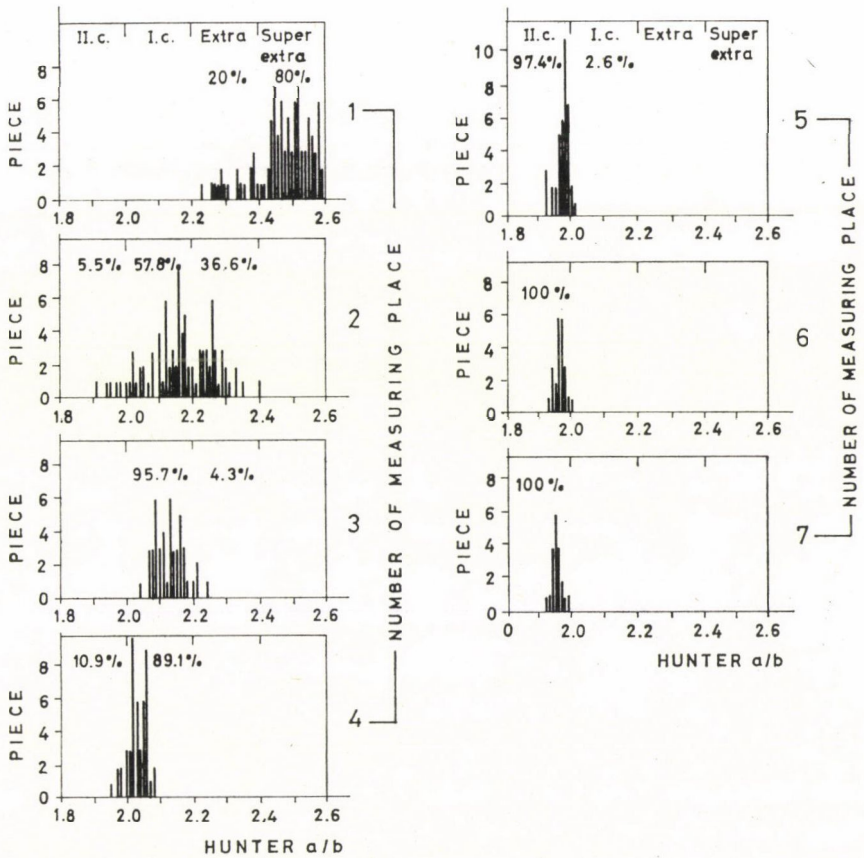

Fig. 5. Effect of processing phases on colour quality distribution (mean values)

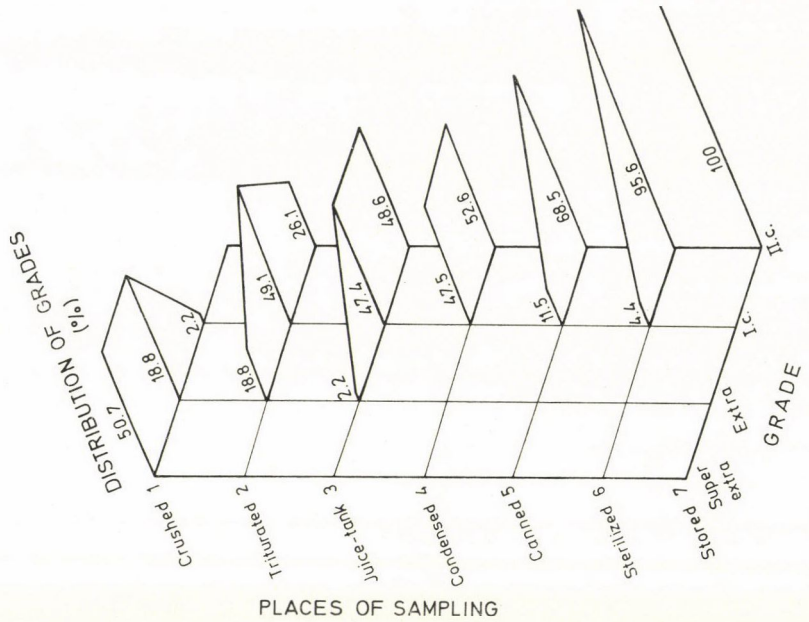

Fig. 6. Percentage distribution of colour development in processing phases

Sterilization and cooling gave 100% class II products.

No practical change was found after 2 months of storage.

At the second measurement, 29% super extra and 71% extra raw materials yielded 13% class I paste. The 13% class I product changed entirely into class II after sterilization and storage.

At the third measurement where raw material quality was identical with that of the second measurement, 98% class II juice was found in the juice tank sample in front of the evaporator.

Colour loss to such an extent could only be explained by drastic heat damage. The cause of it was found in a defective pre-heater. Further processing modified colour only slightly without considerable reduction.

In Fig. 7, average colour values of the different sampling points are shown in L, a–b colour space by the average of the 3 measurements.

The height of columns at the intersection of a–b coordinates is proportional to the L value representing grades of lightness.

In the columns, mean a/b ratio values are shown, on the connecting lines ΔE values indicating actual colour differences.

When analysing the figures, the largest colour difference is found between the raw material and the strained juice ($\Delta E = 1.86$) as the average of the 3 processing lines.

Considerable colour deterioration is found between the strained juice and that of the tank in front of the evaporator ($\Delta E = 1.41$). The colour difference between strained juice and paste amounts to 1.00 ΔE.

Evaporator yield, pasteurization and filling caused only a difference in totalled mean of 0.83 which is not much.

The relatively large decrease after sterilization indicates inadequate cooling.

Some interesting part differences are also found between measurements.

While in the first and third measurements colour difference between the strained juice and paste had the same $\Delta E = 0.46$ value, in the second measurement it was hardly half as much ($\Delta E = 0.91$).

Colour value decrease between cold pulp and paste totalled −0.47 a/b, 4.35 E.

Figure 8 represents a/b colour difference values of the 3 measurements. The Figure as well as Tables 1 and 3 indicate the extent of colour change between two sampling points. At the same time, they reveal at which point of the line undesirable manipulations occur during processing. Excess heat or prolongation of processing caused probably the extreme colour changes which – in the present case – always meant colour deterioration.

The Figure also indicates that process phases have to be supervised in order to restrict or eliminate colour deterioration.

For that purpose, as much as possibilities allow, processing time ought to be shortened, temperature decreased and large temperature differences avoided until evaporation is finished, or it must be followed by drastic cooling

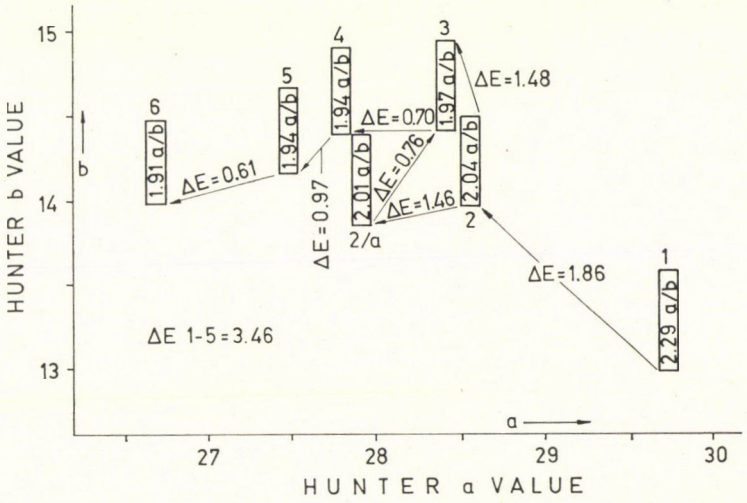

Fig. 7. Mean colour quality and colour differences in processing phases on *Lab* colour diagram

Table 1

Mean a/b and ΔE_{Lab} values in different processing phases and their changes between subsequent operations

		1 pulp	2 strained juice	1–2	3 juice tank	2–3	4 evaporated paste	3–4	5 filled paste	4–5	6 sterilized paste	5–6	7 stored paste	6–7
a/b	1	2.47	2.16	−0.31	2.13	−0.03	2.03	−0.10	1.96	−0.07	1.96	±0.00	1.95	−0.01
	2	2.32	1.98	−0.34	1.98	±0.00	1.99	+0.01	1.98	−0.01	1.95	−0.03	1.94	−0.01
	3	2.37	2.10	−0.27	1.91	−0.19	1.90	−0.01	1.87	−0.03	1.87	−0.00	1.86	−0.01
ΔE_{Lab}	1			1.82		1.47		0.64		0.64		1.56		0.24
	2			2.61		1.40		—		0.76		1.28		0.98
	3			2.18		1.35		1.35		0.23		0.50		0.65

Table 2

Changes of colour characteristics as affected by modification in the processing operations

Colour characteristics	Measurement	1–2 pulp-strained juice	1–3 pulp-juice tank	1–4 pulp-evaporated paste	1–5 pulp-filled paste	1–6 pulp-sterilized paste	1–7 pulp-stored paste
a/b	1	2.47	2.16	2.13	2.03	1.96	1.96
	2	2.32	1.98	—	1.99	1.98	1.95
	3	2.37	2.10	1.91	1.90	1.87	1.86
ΔE_{Lab}	1	1.82	2.47	2.76	3.08	3.84	4.02
	2	2.61	3.26	—	3.36	3.35	4.02
	3	2.18	3.50	4.09	4.12	4.50	5.02

Table 3

Colour values between raw material and stored paste measured on the processing line

Number of measurements	Total colour difference a/b 1–7	Total colour difference ΔE_{Lab} 1–7
1	−0.52	4.02
2	−0.37	4.02
3	−0.51	5.02
Mean	−0.47	4.35

Above all, steps must be taken to receive and process only raw material of good microbiological quality. We are convinced that hot water sterilization, now an integrated part of home technology, is only needed for small filling units. After-treatment could be omitted and replaced by effective cooling by means of cold water spraying for units larger than 1/1.

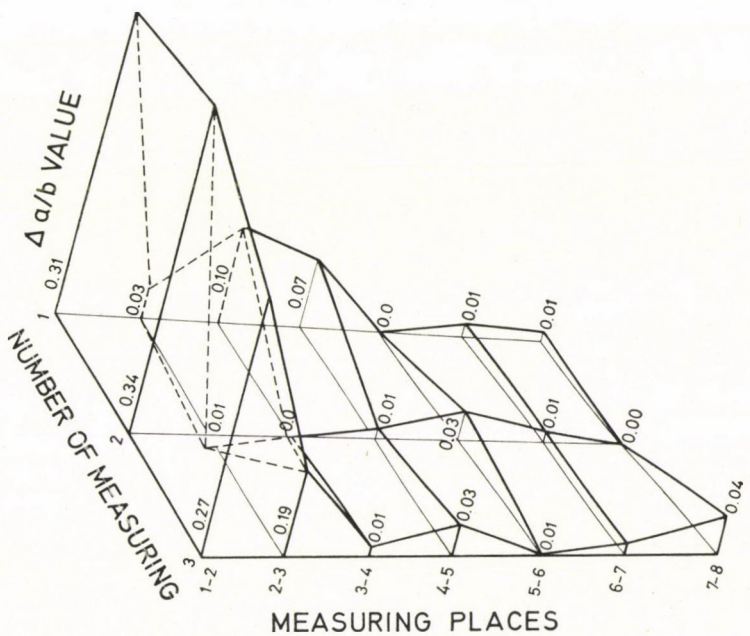

Fig. 8. Evolution of $\Delta a/b$ values between different sampling points to illustrate colour changes; 1–2–3 repeated measurements; 1–2, 2–3, *etc.* colour differences between sampling points

In Fig. 9 results of the same measurement are given in ΔE_{Lab} values. Colour changes between processing phases is represented. Operations resulting in reducing colour can be well discerned.

Depending on processing, different colour value evolutions were found in the a/b measurements.

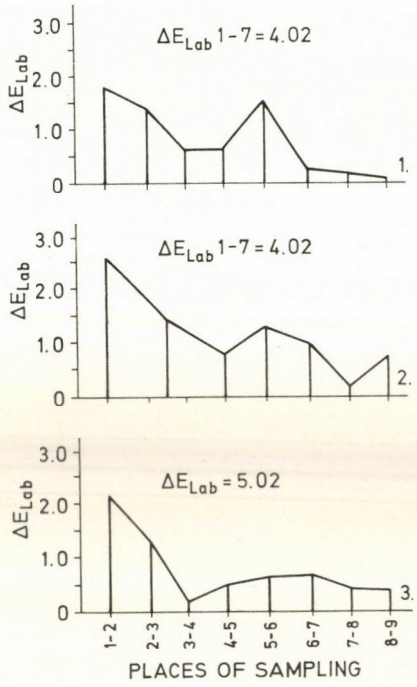

Fig. 9. Evolution of ΔE_{Lab} colour difference values between sampling points of the 3 measurements

Beyond doubt the first and most important colour deterioration took place in the first phase, hot straining: a/b values of –0.30, –0.34, –0.27.

During buffering of the strained juice, the hot material was again damaged, the extent of which varied in the 3 measurements: a/b values of –0.03, $\pm$0.00 and –0.19. It is remarkable that no damage was observed in the second measurement when the second buffer tank was not used and the juice line was joined directly to the vacuum evaporator. At the same time, in measurement 3, with the *Zsigmond* type pre-heater, considerable damage of a/b value –0.19 was found.

Colour changes between buffered juice and paste came up to –0.10, $\pm$0.0 and –0.01 a/b values. After evaporation –0.43, –0.33 and –0.47 a/b values indicated deviation from pulp colour.

Subsequent colour deterioration worth mentioning was only found in the heating-filling process following evaporation with –0.09, –0.01 and –0.03 decrease in the a/b value.

Sterilization did not affect the a/b index considerably (–0.02, –0.03 and –0.01). However, if ΔE_{Lab} values indicating actual colour changes are considered, more important modification is found. Sterilization resulted in changes of 0.18, 0.68 and 0.50 ΔE.

For the whole processing line, data of the 3 Tables give numerical information concerning a/b and ΔE values from sample 1 (pulp raw material) to sample 7 (paste stored for 2 months).

It appears that in respect of a/b colour indexes results of the first and third measurements are practically the same (–0.50), corresponding to 2 1/2 colour class diminution while the second measurement with its relatively smaller a/b value (–0.37) corresponds to not fully 2 colour class deterioration.

In respect of ΔE_{Lab} spatial colour differences, the case is somewhat different. Measurements 1 and 2 have ΔE values of 4, while measurement 3 has 5, deviating by an entire sensory grade.

At the same time, at pasteurization prior to filling the second measurement had a colour difference of 1.1 on the average; the other two only 0.48 and 0.51, respectively.

Considerable difference was found in colour also after sterilization. In the first and second measurements, 1.25 and 1.16 values were obtained, in the third only 0.51.

4. Conclusions

Studies raise two fundamental questions:

– How large a loss source should be taken into consideration in up-to-date evaporator lines to secure the production of class I products from the raw material available? The literature does not contain any reliable data. According to our present and previous, independent measurements a colour deterioration of an a/b value of 0.40–0.50 must be expected during processing by common technology.

Accordingly, colour deterioration corresponding to two and a half colour grades can be reckoned with between the cold pulped and the hot pulped product.

– What minimum colour value is required in the raw material to obtain colour class I product? As shown, the rate of average colour deterioration is 0.50 a/b ratio. If we want to produce paste superior to an a/b value of 2.0, raw material of 2.5 a/b, at least, is needed.

There are other possibilities to optimize certain operations (pre-heating, straining, evaporating, sterilizing, *etc.*) so as to have a more favourable impact on colour modification than those used in our studies.

Because of its complexity the problem needs further studies.

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ASSAY INTO THE PROTEIN-LIPID COMPLEXES OF WHEAT FLOUR SOLUBLE IN PETROLEUM ETHER

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The composition and the interrelations of protein and lipid were investigated in the protein–lipid complex isolated from the petroleum ether extract of wheat flour.

Various methods were tested for the isolation of the complex without degradation and maintaining the lipoprotein formed *in vivo*. The apoprotein and lipid parts of the isolates were analysed.

The petroleum-ether-soluble lipoprotein was found to be an unstable complex liable to degradation the composition of which, that is the quality and quantity of lipids complexing the protein, is largely dependent on the mode of isolation and the age of the isolate.

By means of analysis of the isolates and model experiments, carried out with the original and modified apoprotein fractions, respectively, the occurrence of three types of protein–lipid interaction could be proved.

A part of the phosphatides containing free amino groups is bound to the protein component by double salt-like bond; this stable protein–lipid complex contains an amount of phosphatide equivalent with the amount of the amino acids of basic or acidic side-chain sequentially adjacent in the apoprotein.

The second part of lipids is bound more weakly, only by one salt-like bond to the basic side-chains of the apoprotein.

The neutral lipids enter into interaction with the orientationally placed phosphatides and with the hydrophobic region of the apoprotein, resp., forming an unstable complex which is liable to degrade in the course of isolation by solvent precipitation.

The compositional proportions of the complex described in the literature as lipopurothionin and obtained by precipitation in methyl acetate, as well as of the complex containing the complete neutral lipid fractions were established in the course of this study as well as the ratio of the protein fractions and the amount of lipids entering into interaction with the former.

The study of protein–lipid complexes is one of the most dynamically developing branches of biochemistry. In addition to the explanation of explicitly theoretical problems, the knowledge of the structure and characteristics of protein–lipid complexes has become decisively important in various practical areas (clinical chemistry, food science) because of the crucial importance of these compounds in biological processes and of their importance in food technology.

As was established in an earlier paper (BÉKÉS, 1977), lipoproteins play an important role in physiology and food technology, even if this is not elucidated in every aspect. Beside the protein–lipid complexes which are

formed in the course of bread making and strongly influence the rheological characteristics of dough, the purothionin–lipid complex, probably interreiated already *in vivo*, and isolated from the petroleum ether extract of wheat flour, has been thoroughly studied.

Purothionin, first isolated by BALLS and HALE (1940) stands in the forefront of interest, due to the unusual amino acid composition (high lysine, arginine and cysteine contents) and to its hitherto unknown biological and biochemical functions and toxicity to microorganisms.

In addition to the activity of those who first isolated them, several research teams (BALLS *et al.*, 1942a, b; BALLS & HALE, 1944; COULSON *et al.*, 1942; STUART & HARRIS, 1942) were engaged in elucidating problems related to the separation, physical and chemical characteristics, toxicity of purothionin (FISHER *et al.*, 1968; REDMAN & FISHER, 1968, 1969; AXFORD *et al.*, 1968; REDMAN & EWART, 1973; NIMMO *et al.*, 1968, 1974; CARBONERO & GARCIA-OLMEDO, 1969; FERNANDEZ DE CALEYA *et al.*, 1972; PATEY *et al.*, 1976; OKADA *et al.*, 1970a, b, 1973; RAO *et al.*, 1978; OKADA & YOSHIZUMI, 1976). Research on the purothionin content of Hungarian wheat varieties has been carried out for several years at this Department (LÁSZTITY *et al.*, 1969, 1978a, b, 1979; BÉKÉS *et al.*, 1975, 1976; BÉKÉS, 1976, 1977; VARGA *et al.*, 1975). The two low-molecular fractions of purothionin (α- and β-purothionin) form the first wheat protein of non-enzymic character the amino acid sequence of which is known. The sequences were established by MAK and JONES (1976, 1977) and OHTANI and co-workers (1975) independently from one another.

In contrast to the extensive literature on apoprotein, relatively little is known of the lipid complex of purothionin, called lipopurothionin by REDMAN and FISHER (1968). The isolation method, based on precipitation from methyl acetate was developed by REDMAN and FISHER (1968) and they determined the main components of the lipid part of the complex. HOSENEY and coworkers (1970, 1971) isolated the lipopurothionin from wheat by precipitation first from butanol, then from acetone and finally from ethyl acetate and used the same technique to isolate the barley protein analogous to purothionin, named hordothionin. Spanish researchers studied lipopurothionin and particularly the glycolipid components in relation to solubility (FERNANDEZ DE CALEYA *et al.*, 1976; HERNANDEZ-LUCAS *et al.*, 1977). The lipids of lipopurothionin, in addition to the sterol ester present in trace amount, are polar, in the majority phospholipids (phosphatidyl ethanolamine, phosphatidyl cholin) and there are two glycolipids (digalactosyl diglyceride, monogalactosyl diglyceride). The aim of the present study was the qualitative and quantitative characterization of the lipid complexes of purothionin, in other words the determination of the stoichiometric composition (proportion of the lipid and protein components) and the investigation of the interaction of these components.

As it is stressed in every work concerned with lipopurothionin, the main difficulty of analysing the complex lies in its isolation: the separation of the "free" lipids without interfering with the composition of the protein–"bound lipid" complex. In the course of this study the authors succeeded in isolating the complex by methods milder than precipitation from a solvent and thus it was possible to compare the composition of the complexes obtained by different methods.

The nature of the interaction of the protein–lipid complexes and the state of the active amino acid side-chains participating in the bonds were studied in model experiments when the apoprotein fraction of the complex and its derivatives, produced by chemical modifications, were brought together with lipids.

1. Materials and methods

1.1. Materials

In the experiments the mixed wheat flour Bl–112, commercialized for bread manufacture of maximum 1.12% ash content, was used.

1.2. Production and fractionation of protein–lipid complexes

The complexes were obtained from the petroleum ether extract of wheat flour. Extraction was carried out by the method of one of us (BÉKÉS, 1977).

Four kinds of preparations were obtained from the petroleum ether extract:

— purothionin and its fractions by treatment with hydrochloric acid according to BALLS and HALE (1940) or by its variant, modified by one of us (BÉKÉS, 1977);

— lipopurothionin by the methyl acetate technique developed by REDMAN and FISHER (1968);

— purothionin "contaminated" by lipid by applying *Folch*'s washing to lipopurothionin;

— purothionin–lipid complex by gel permeation fractionation of the petroleum ether extract.

The scheme of isolation of the preparations is shown in Fig. 1.

1.2.1. Preparation and fractionation of lipopurothionin. Twenty g of the petroleum ether extract were dissolved in 200 cm^3 methyl acetate. This resulted in a white flocculent precipitate. The mixture was cooled in ice-water for 20 min, then centrifuged for 15 min at 1 000 rpm and washed twice in 50 cm^3 methyl acetate. The clear supernatant was used to determine the "free lipids".

The raw preparation was purified as follows: the complex was dissolved in 20 cm^3 petroleum ether and then it was precipitated with 50 cm^3 methyl

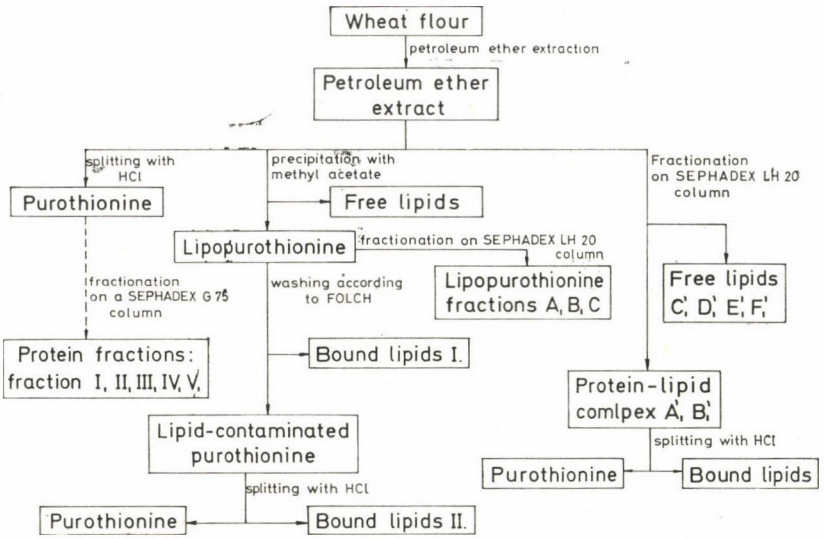

Fig. 1. Scheme of the protein–lipid complexes isolated from the petroleum ether extract of wheat flour and of the isolation of apoproteins and bound lipids. The isolated substances and their fractions are shown as marked in the text

acetate. The precipitate was then dissolved in 15 cm³ petroleum ether and then precipitated with methyl acetate added drop by drop. The mixture was allowed to stand for 1 h below −5 °C. After centrifuging the precipitate was dried above $CaCl_2$.

Fractionation of lipopurothionin was carried out on a *Sephadex* LH 20 column according to FISHER (1970).

The petroleum ether extract of wheat flour was also fractionated by the same method. Detection of the eluates and collection of the fractions was carried out on a *LKB Uvicord* automated fraction collector at 260 nm. For measuring the absorption at 240 nm of the 3 cm³ fractions, a *Spectronom* 202 apparatus was used.

1.2.2. Breaking down of the protein–lipid complexes by washing according to Folch. From lipopurothionin, from its fractions and the fractions obtained by direct fractionation of the petroleum ether extract 10 mg each were dissolved in 1 cm³ of the 2 : 1 mixture of chloroform and methanol. To this solution a quarter volume of 0.88% aqueous potassium chloride was added. The mixture was shaken for 1 h and the two phases formed were separated: in the upper phase, rich in water, the apoprotein part of the protein–lipid complex, while in the lower phase, rich in chloroform, the "bound lipid" fraction I, was obtained.

1.2.3. Purification with hydrochloric acid of the apoprotein fractions isolated by washing according to Folch. The fractions obtained by washing according to *Folch* were not free of lipids. To remove the lipids an analogy of the

classical method, used to separate purothionin (BALLS & HALE, 1940) was applied: cleavage in the absolute alcohol solution of hydrochloric acid. Fifty mg protein obtained by the *Folch* washing procedure were suspended in 7 cm^3 absolute ethanol then 1 cm^3 diethyl ether and 1 cm^3 10 *N* hydrochloric acid reagent were added. (The hydrochloric acid reagent is dried HCl gas absorbed in absolute ethanol after liberation from conc. hydrochloric acid by the addition of conc. sulfuric acid.)

The mixture was shaken for 15 min then the white precipitate was separated from the yellowish solution by centrifuging at 6 000 rpm for 15 min. The pure apoprotein was finally obtained by washing the precipitate in absolute ethanol then in the 1 : 1 mixture of absolute ethanol and diethyl ether. In the supernatant "bound lipid" fraction II, was obtained.

1.3. Analysis of the isolated preparations

All the preparations shown in Fig. 1 were exposed to qualitative and quantitative analysis. In order to relate the preparations to the original material, the individual preparations were weighed, nitrogen and phosphor and in the products of protein character the protein content were determined.

The composition of the lipid fractions was studied by thin-layer chromatography. This was in certain cases complemented by the gas chromatography of the total fatty acid composition of the given fraction.

Every apoprotein isolate was analysed by polyacrylamide gel electrophoresis and the results were quantitatively evaluated. In some cases the protein fractions were further fractionated according to molecular size and the subfractions thus obtained were exposed to gross amino acid analysis and to the determination of their *N* and *C*-terminal amino acids.

Determination of the nitrogen and phosphor content, the gas chromatography and the characterization of the protein fractions were carried out by the methods as described in an earlier paper (BÉKÉS, 1977). The other methods applied are described below.

1.3.1. Determination of the protein content. The protein content of isolated apoproteins was determined on the modul based on the *Folin* reaction on *Contiflo* automated serial analyser (manufactured by LABOR MIM, Hungary). The modul applied, the composition of reagents and data of the volume-flow were taken from the paper of VARGA and co-workers (1976). Quantitative evaluation was based on a calibration curve constructed with purified purothionin.

1.3.2. Thin-layer chromatography of the lipid fractions. The "free" and "bound" lipid fractions were studied on hand-made and industrially manufactured *Kieselgel* G layers (*Silufol*, made in Czechoslovakia; *Servacel*, product of SERVA). For development various solvents and spray reagents were used.

After testing a number of solvents, finally two systems were selected. Apolar lipids were run to $R_f = 0.5$ with the solvent mixture diethyl ether–benzene–absolute ethanol– acetic acid (40 : 50 : 2 : 0.2) and after drying they were run to the end of the layer in a diethyl ether–hexane mixture (6 : 94) (MORRISON, 1970). For the polar lipids, the thin-layer was first defatted with the 3 : 1 mixture of petroleum ether–diethyl ether. The lipids were then run in chloroform–methanol–30% ammonium hydroxide–water (60 : 35 : 5 : 2.5).

The spots were sprayed with 50% sulfuric acid developing reagent or reagents specific for the different lipid classes, *e.g. Zinzadze* reagent for phosphatides, ninhydrin for the detection of amino groups, *Dragendorf* reagent for methylated amines and the orcin reagent to identify glycolipids. The reagents were prepared and applied according to KATES (1972).

Identification of the spots, the qualitative analysis of lipids was carried out by comparing to lipid standards manufactured by SERVA, to the data with R_f values taken from the literature and by using the information obtained from group-selective reagents.

For quantitative evaluation, determination of the gross composition of the sample applied after treatment with sulfuric acid reagent and total carbonization, a *Vitatron* type densitometer was used. The relative error of lipid determination by thin-layer chromatography amounted to 10–15%.

1.4. Model experiments to study protein–lipid interaction

By adding appropriately prepared aqueous lipid dispersions to the aqueous solution of purified purothionin or its homogeneous fractions according to molecular size, isolated on *Sephadex* G 75 column (BÉKÉS, 1977), the inclination of pure lipid preparations and wheat lipid fractions to form precipitate, was compared. Thus by changing the proportion of protein to lipid, information may be obtained on the inclination of the components to interact and on the optimum proportion of the components in the complex. By appropriate chemical modification of the protein components, the role played by individual amino acid side-chains in complex formation may be elucidated.

These model experiments were based on the papers of DAS and CRANE (1963) and BRAUN and RADIN (1969).

1.4.1. Description of the model experiments. The 10 mg cm^{-3} chloroform solutions of the lipids used in the experiments were dried by cold ventilation. The residue was dispersed with water sufficient to obtain a lipid content of 0.5 or 2.0 mg cm^{-3}. Dispersions were obtained in a *Labaid* blender (made in Poland) fitted with a special stirring head. The protein solutions were of 0.4 mg cm^{-3} concentration. The model mixture contained 2.0 cm^3 protein solution and lipid dispersion of varied volume as well as distilled water to have a final volume of 7.0 cm^3. In some of the experiments distilled water was

replaced by solutions of appropriate pH or the solution of Na, Ca or Al as required. The united components were allowed to stand for 15 min at 25 °C. The mixture was then centrifuged at 3000 rpm on a table centrifuge (a *Janetzky* product). The protein and lipid contents of the supernatant were analysed paccording to para. *1.3.* In addition, the properties of the precipitated model complex were tested by ultracentrifuging and by their UV spectra (see para. *1.4.3.*).

In analysing the supernatant the quantity of lipid related to unit protein or $\overline{\vartheta}$ (initial lipid concentration minus lipid concentration upon precipitation) was plotted as a function of the initial quantity of lipid, as described by MALCOLM (1975).

The complexing reaction of egg lecithin (MERCK) and lipid standards of SERVA (L-α-dipalmitoyl lecithin, phosphatidyl-ethanolamine, phosphatidyl inositol, phosphatidyl serine, lysophosphatidyl-ethanolamine, cholesterin, ergosterin-palmitate, monogalactosyl-diglyceride, digalactosyl-diglyceride), or their mixtures of given proportion with purothionin or its fractions of low molecular mass (IV–V) and the derivatives of these fractions masked by maleic acid anhydride or cyclohexandion, was studied (see para. *1.4.2.*).

1.4.2. Preparation of purothionin fractions chemically modified. The method of BUTLER and co-workers (1969) was applied to modify the basic side-chains of amino acids with maleic acid; the method of PATTY and SMITH (1975) was used to block reversibly the arginyl groups with cyclohexandion.

1.4.3. Analysis of model complexes. The analysis of the complexes isolated in the course of the model experiments were studied on the analytical ultracentrifuge, Type 120 manufactured by MOM (Hungary). The homogeneity testing according to molecular size and the determination of the sedimentation constants were carried out from the chloroform solutions (0.5–1.4%) at 30 000 or 50 000 rpm and 25 °C, by using *Schlieren* optics.

The structural properties of the protein and lipid samples used in the experiments and the structural analogies of the model and isolated complexes were followed up in their UV spectra. The spectra were recorded on the UV *Specord* apparatus (manufactured in the GDR), under thermostating at 25 °C *versus* solvent, in the wavelength region of 230 to 310 nm.

Results

2.1. Isolation and analysis of lipopurothionin

Of 20.0 g petroleum ether extract of wheat flour 0.85–0.92 lipopurothionin could be isolated. This amounts to 0.370–0.375 g per 1 kg wheat flour.

2.1.1. Results of fractionation experiments. The elution diagram of lipopurothionin on *Sephadex* LH 20 column in the 2 : 1 mixture of chloroform–

methanol, subsequently in the 1 : 1 mixture of chloroform–methanol, is shown in Fig. 2.

The elution diagram in the Figure was plotted immediately upon isolation. It was established in the course of this study that the isolated lipopurothionin, particularly when dry, changes substantially, the fractions of larger molecular size become enhanced. In Fig. 3 the elution curve of a lipopurothionin sample, stored in the dry state for 14 days at 0 °C, is shown.

Further studies were carried out always from freshly prepared preparations.

From the solutions obtained by fractionation main fractions A, B and C, as shown in Fig. 2, were separated.

2.1.2. Chemical composition of lipopurothionin and its fractions. Table 1 contains the main data of fraction distribution in lipopurothionin and those of the protein and lipid components separated by washing according to *Folch*.

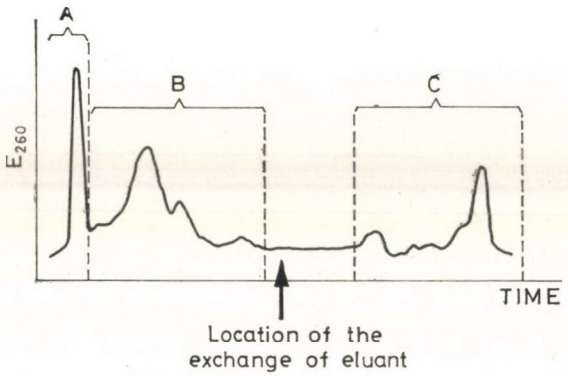

Fig. 2. Elution curve of freshly isolated lipopurothionin. Column: *Sephadex* LH 20 (52×2.6 cm). Eluents: chlroform–methanol (2 : 1) then chloroform–methanol (1 : 1). The collection intervals of the three main fractions are given

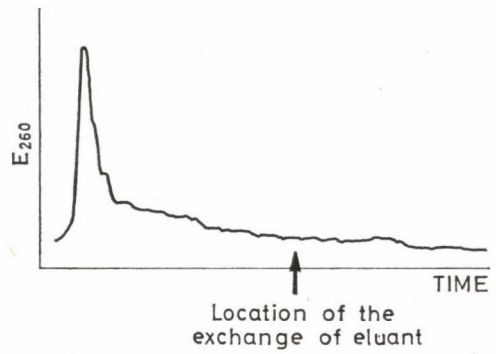

Fig. 3. Elution curve of lipopurothionin isolate stored for 14 days at 0 °C. For conditions of elution see legend to Fig. 2

Table 1

Main data of the composition of lipopurothionin and its fractions

	Lipopuro-thionin	Main fractions		
		A	B	C
Distribution of fractions (%)	100.0	41.2	32.3	26.5
Protein content (%)	27.4	30.4	26.4	25.7
Lipid content (%)	72.6	69.6	73.6	74.3
Phosphorus content ($\mu g\ mg^{-1}$)	20.0	21.3	18.4	19.2

Out of the elution curves obtained on *Sephadex* G 75 column from the upper phase, rich in water, of *Folch* washing and of the gel electropherograms two curves each are shown in Figs. 4 and 5. For the sake of comparison the curves of purothionin obtained under similar conditions by the method of BALLS and co-workers (1942) are also given.

The distribution of fractions in apoproteins is summarized in Table 2.

Table 2

Distribution of apoprotein fractions isolated from lipopurothionin

Apoprotein (purothionin) fractions	Lipopuro-thionin	Main fractions		
		A	B	C
I.	49.8	49.8	41.3	19.7
II.	17.3	28.7	33.6	46.5
III.	18.3	6.9	7.2	9.7
IV. + V.	14.6	14.6	17.9	14.1

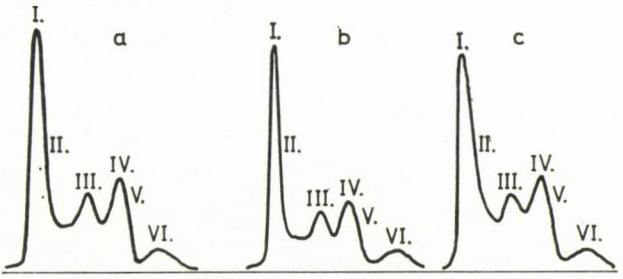

Fig. 4. Elution curve of the apoprotein fraction. *a:* Lipopurothionin; *b:* lipopurothionin fraction A; *c:* purothionin isolated according to BALLS and co-workers. Column: *Sephadex* G 75 (22 × 2.2 cm); V_0 = 27 cm³; Eluent: Acetic acid 0.05 *M* + aqueous 0.01 *M* KCl solution

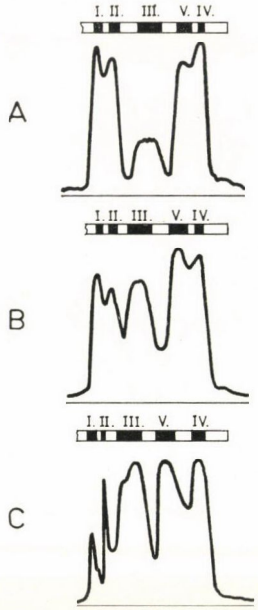

Fig. 5. Electropherogram of the apoprotein fraction. *a:* Lipopurothionin; *b:* lipopurothionin fraction A; *c:* purothionin isolated according to BALLS. For experimental conditions see: BÉKÉS (1977)

Results on lipopurothionin components obtained by the densitometry of thin-layer chromatograms from the lower phase after *Folch* washing, are contained in Table 3.

In different experiments the protein preparation isolated from the upper phase in *Folch* washing proved to be contaminated. Its solubility in water

Table 3

Composition of lipopurothionin and its fractions (Bound lipids I) as percentage of total lipid content

Lipid fraction	Lipopuro-thionin	Main fractions		
		A	B	C
Sterolester	1.64	1.68	1.74	1.49
Sterylgylcoside	1.84	1.86	1.90	1.77
Phosphatidyl-ethanolamine	36.88	39.73	41.72	28.23
Phosphatidyl-cholin	30.49	24.83	31.94	36.60
Phosphatidyl-inositol	5.23	3.42	4.09	8.80
Phosphatidyl-serine				
Lysophosphatidyl-ethanolamine	0.80	0.39	0.78	1.30
Monogalactosyl-diglyceride	7.86	9.20	6.54	7.41
Digalactosyl-diglyceride	14.42	15.90	12.48	14.40
Monogalactosyl-monoglyceride	0.84	3.02	4.10	traces

was found to be lower than that of purothionin obtained by the traditional method and its behaviour in precipitation experiments proved to be anomalous. In detailed analyses a phosphorus content of 0.17–0.21 $\mu g\ mg^{-1}$ was found.

On purification by cleavage of the still bound lipids with hydrochloric acid, both solubility and ability to precipitate improved to the level of that of purothionin.

Results of analyses of the lipid-containing supernatant of hydrochloric acid cleavage are shown in Table 4 (Bound lipids II).

Table 4

Data of the lipid fraction (Bound lipids II) isolated from lipopurothionin in the course of purification of purothionin with hydrochloric acid

Purothionin contaminated by lipid	
Phosphorus content ($\mu g\ mg^{-1}$)	1.7
Protein content (%)	92.5
Lipid content (%)	7.5
Gross composition of lipids	**%**
Phosphatidyl-ethanolamine	28.2
Phosphatidyl-serine	26.1
Digalactosyl-diglyceride	35.3
Monogalactosyl-diglyceride	10.4

2.2. Direct fractionation and analysis of the petroleum ether extract

The elution curves of fractionation of the petroleum ether extract on *Sephadex* LH 20 column are shown in Fig. 6. In washing the isolated 6 fractions according to *Folch*, of the upper phases rich in water only two, those marked A' and B' gave positive protein reaction.

The mass ratios and data of composition are contained in Table 5.

Based on gel electrophoretic behaviour and gross amino acid composition, the protein components of fractions A' and B' unambiguously correspond to the purothionin fractions. The proportions of the fractions in the protein preparation isolated from the A' and B' fractions, resp., of the lipid–protein complex, do not differ significantly from the data given in Table 2 for the apoproteins of lipopurothionin.

The lipid composition of the six fractions obtained by *Folch* washing in the phase rich in chloroform, are summarized in Table 6.

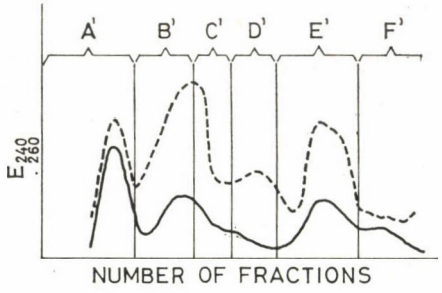

Fig. 6. Direct fractionation of the petroleum ether extract on *Sephadex* LH 20 column. For conditions of isolation see legend to Fig. 2. The solid line stands for extinction at 260 nm; the dashed line for 240 nm

Table 5

Characteristics of fractions of the petroleum ether extract of wheat flour fractionated on Sephadex *LH 20 column*

	Fractions					
	A′	B′	C′	D′	E′	F′
Distribution of fractions as % of the material applied	11.7	53.5	11.7	15.0	3.3	5.5
Protein content (%)	14.9	10.6	—	—	—	—
Lipid content (%)	85.1	89.4	—	—	—	—
Phosphorus content ($\mu g\ mg^{-1}$)	12.8	8.8	5.6	4.4	4.7	3.8

Table 6

Lipid composition of fractions obtained by direct fractionation of the petroleum ether extract of wheat flour

Lipid fractions	Extract fractions					
	A′	B′	C′	D′	E′	F′
	as % of total lipid					
Free sterol	—	—	8.6	10.1	8.8	8.7
Sterol ester	4.8	7.8	16.4	19.1	15.4	5.4
1,2 diglyceride	—	—	8.1	10.2	11.4	4.1
1,3 diglyceride	—	—	11.8	9.6	7.3	4.0
Triglyceride	18.7	60.0	29.4	29.1	33.8	65.0
Free fatty acid	—	—	6.3	5.8	5.9	3.2
Polar lipids	46.5	32.2	19.2	16.1	17.4	13.7

The distribution pattern of polar lipids in fractions A' and B' containing also protein is given in table 7.

Table 7

The polar lipids of two protein containing fractions obtained by direct fractionation of the petroleum ether extract of wheat flour (in percentage of total lipid)

Lipid fraction	Fraction	
	A′	B′
Phosphatidyl-ethanolamine	16.2	12.7
Phosphatidyl-cholin	18.9	10.0
Phosphatidyl-inositol	0.3	1.0
Phosphatidyl-serine		
Lysophosphatidyl-ethanolamine	0.8	0.4
Monogalactosyl-diglyceride	3.9	2.6
Digalactosyl-diglyceride	6.0	5.2
Monogalactosyl-monoglyceride	0.4	0.3
Total of polar lipids	46.5	32.2

2.3. Comparison of the gross fatty acid composition of "free" and "bound" lipid fractions

The results of gas chromatographic fatty acid analysis of the petroleum ether extract, of its fractions (A'–B') isolated on *Sephadex* LH 20 column, of the supernatant containing methyl acetate subsequent to the precipitation of lipopurothionin ("Free lipids"), of lipopurothionin, of its fractions, are given in Table 8.

2.4. Results of model experiments

2.4.1. Model experiments with purothionin. The precipitation model experiments with purothionin isolated according to BALLS and co-workers (1942) permit of the following conclusions:

— Purothionin formed a model complex with each of the phospholipids studied. Fundamentally, the $\overline{\vartheta}$–A diagrams show two patterns: those of lecithin and phosphatidyl inositol are of a simple saturation curve character, while those of phosphatidyl-ethanol amine and phosphatidyl serine are of two-step saturation curve character (Fig. 7). The final saturation $\overline{\vartheta}$ value of all the lipids proved to be identical.

Table 8

Comprehensive table of the fatty acid composition of "free" and "bound" lipid fractions

Fatty acid	14 : 0	16 : 0	16 : 1	16 : 2	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0
	in percentage of total fatty acid content								
Petroleum ether extract	0.1	23.3	0.19	trace	1.6	22.6	46.7	5.0	1.1
Fraction A′	0.6	31.6	2.5	2.6	9.8	42.4	12.3	trace	trace
Fraction B′	0.8	28.2	2.8	0.8	9.6	43.2	12.1	1.7	0.8
Fraction C′	0.1	23.3	0.2	trace	1.7	22.8	46.3	4.4	1.1
Fraction D′	trace	23.4	0.2	trace	1.6	22.9	46.7	4.9	1.0
Fraction E′	0.1	20.9	trace	trace	3.0	19.5	49.3	4.4	1.4
Fraction F′	0.1	23.7	0.2	trace	1.0	21.2	46.6	3.0	1.0
"Free lipids"	trace	18.6	0.18	trace	0.74	14.1	61.7	2.7	0.5
Bound lipid I in lipopurothionin	0.8	29.4	2.4	0.4	9.9	42.0	12.1	2.8	0.3
Bound lipid II in lipopurothionin	trace	26.3	2.5	0.2	10.2	45.5	12.2	2.7	0.8
Lipopurothionin									
Fraction A	0.6	31.0	2.5	0.6	9.7	44.1	11.4	trace	trace
Fraction B	0.7	30.8	2.5	0.2	9.8	40.1	12.4	2.7	0.8
Fraction C	0.8	28.2	2.6	0.5	10.1	42.4	12.5	2.9	trace
Bound lipid I									

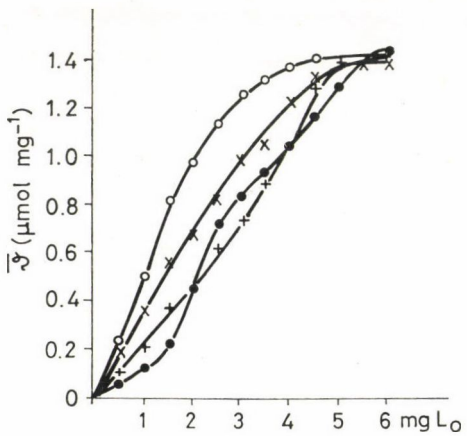

Fig. 7. Saturation curve of purothionin–phospholipid model complex. Final volume: 7.0 cm³; protein content: 0.8 mg. Symbols: —○—○— lecithin; —×—×— phosphatidyl-inositol; — + — + — phosphatidyl-serine; — ● — ● — phosphatidyl-ethanolamine

(The dimension in Fig. 7 of $\bar{\vartheta}$ is not the customary, because of the different molecular mass of the proteins present. The bound lipid molecules were related to the mass of protein weighed in.)

The points in this and the following diagrams represent the average of three, in some cases of five parallel experiments. For the sake of lucidity, standard deviations about the mean were omitted. The coefficient of variation related to the lipid contents in the model experiments did not exceed 15%, which in the case of the values represented corresponds to a maximum of ± 0.3.

Even by varying the protein–lipid proportion within very wide limits was not possible to obtain a model complex from the mixture of purothionin–glycolipid or of purothionin–sterol.

When the above experiments were repeated with purothionin isolated by *Folch* washing, complex formation could not be detected even with phospholipids. These results led the authors to the conception that the isolated preparation contains still bound lipids. As it was described in para. *2.1.2.*, from the isolate obtained by *Folch* washing the lipids still present were separated by purification with hydrochloric acid ("Bound lipids II") and the purified protein thus obtained showed characteristics in model experiments identical with the above.

2.4.2. Model experiments with purothionin fractions of low molecular mass. Model experiments without repeated fractionation were carried out with the main fraction of low molecular mass isolated on a *Sephadex* G 75 column (IV–V). These experiments aimed at the investigation of complex forming reactions in the joint presence of fractions α and β based on the customary nomenclature of the literature. The results may be summarized as follows.

The behaviour of the fractions of low molecular mass to phospholipids is similar to that observed with the complete purothionin. In Fig. 8 the characteristic saturation curves of phosphatidyl ethanolamine and lecithin are illustrated. Here the dimension of $\bar{\vartheta}$ is given in the customary unit (μmol lipid per μmol protein) considering the molecular mass of the protein to be 5000. As it may be seen in the Figure, the final level of saturation corresponds to value 12, while the first platform shown by phosphatidyl ethanolamine (and by phosphatidyl serine which is not illustrated) is 2 mol lipid per protein molecule.

When investigating the pH and ionic strength dependence of complex forming capacity this was proved to be extremely variable with pH and with the amount of certain cations. As shown in Table 9, complex formation is strongly repressed in the acidic pH domain, it is at its highest at neutral pH and is relatively lower in the alkaline pH domain. The optimum is to be found at pH = 6.7. Sodium ions were found by the authors ineffective in complex formation. In the case of calcium ions, a strong inhibitory effect is exhibited

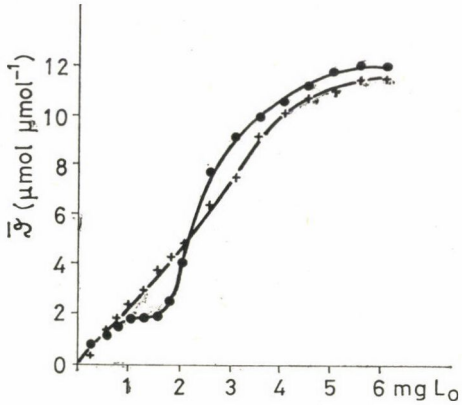

Fig. 8. Saturation curve of purothionin fraction of low molecular mass ($\alpha + \beta$) or (IV + V) model complexes formed with phospholipids. Final volume: 7.0 cm^3; protein content: 0.8 mg. Symbols: —●—●— phosphatidyl-ethanolamine; —+—+— lecithin

Table 9

Model experiments with α and β fractions of purothionin and egg lecithin. Changes in $\bar{\vartheta}$-L correlation as a function of pH and the amount of polyvalent cations

$\bar{\vartheta}$ = (μmol lecithin per μmol protein)

mg	pH = 1.75	pH = 5.1	pH = 6.8	pH = 10.2	Ca^{++} = 0.01	Ca^{++} = 0.001	Al^{3+} = 0.1	Al^{3+} = 0.01	Al^{3+}
4.0	1.0	4.3	10.5	9.8	8.3	6.7	1.9	6.7	8.9
4.5	1.4	6.3	11.4	10.1	8.9	7.4	1.4	6.9	9.4
5.0	2.6	7.1	12.0	11.6	6.9	11.8	4.7	7.0	10.8
5.5	3.9	8.2	12.3	11.9	6.3	12.3	5.1	8.1	11.4
6.0	4.2	9.6	12.0	11.4	7.0	12.7	5.9	8.6	12.2

Ca^{++} at 0.1 concentration total inhibition was observed
Na^{+} significant change was not observed at any of the concentrations studied

and in a 0.1 *N* solution, complex formation stops alltogether. However, it is restored in a solution of 0.01 normality. A similar, though somewhat milder effect is shown by the presence of Al ions.

Model experiments with sterols and sterol esters were negative even with fractions of low molecular mass: no precipitation was observed. In experiments with glycolipids, no complexes were formed either. However, in model experiments with phospholipid–glycolipid mixtures, the glycolipids became integrated in the complex.

This Figure contains the diagrams of experiments carried out with lipid mixtures. As it may be seen the stoichiometry of protein-phospholipid is not influenced by the presence of glycolipids; integration of the glycolipids begins only with relatively higher initial lipid concentrations, the amount integrated

is practically independent from the quality of phospholipid present. The Figure illustrates a 1 : 1 mixture of phospholipid and glycolipid. Experiments carried out with mixtures of different ratio seem to prove that the integration of glycolipids is not so much depending on the protein–lipid ratio as on the phospholipid–glycolipid ratio.

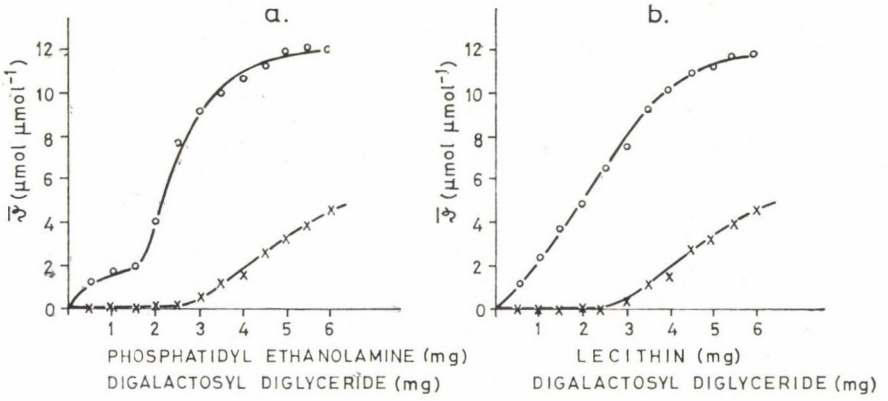

Fig. 9. Saturation curve of low molecular purothionin fraction model complexes formed with phospholipid–glycolipid mixtures. *a:* Phosphatidyl-ethanolamine–digalactosyl-diglyceride (1 : 1); *b:* lecithin–digalactosyl-diglyceride (1 : 1). Final volume: 7.0 cm^3; protein content: 0.8 mg. Symbols: o—o phospholipid, ×—× glycolipid

2.4.3. Model experiments with chemically modified fractions of low molecular mass. The complex formation of proteins could be influenced by chemical modification of the basic side-chains of amino acids. When maleic acid anhydrine was used for masking complex formation was not observed. When the arginyl side-chains were blocked the results seen in Fig. 10 were obtained: the amount of phospholipids entering in interaction with the protein (the phosphatidyl-ethanolamine experiment is illustrated) is significantly lower in the case of masked protein than with the original protein, or after the removal of the masking agent. In the case of phospholipids yielding two-step saturation curve $\overline{\vartheta}$ values change from 1.12 to 1.4.

2.4.4. Comparison of protein lipid complexes isolated and obtained from model experiments. The lipopurothionin obtained by precipitation with methyl acetate and the purothionin–lecithin complex precipitating in the course of model experiments correspond in their behaviour when ultracentrifuged and in their UV spectra.

In Fig. 11 the photos of some ultracentrifuge experiments are shown. The inhomogeneity according to molecular size of both the lipopurothionin and the model complex is well visible. The sedimentation constant related to the main fraction of lipopurothionin is: 6.47 *Svedberg.*

Table 10

Composition of model complexes of ($\alpha + \beta$) fraction of purothionin formed in various phospholipid–glycolipid mixtures

L_0 (mg)		$\bar{\vartheta}$ glycolipid (μmol μmol^{-1})
Phosphatidyl-ethanolamine	Monogalactosyl-diglyceride	
3	6	3.5
6	6	5.6
9	6	6.7
Phosphatidyl-ethanolamine	Digalactosyl-diglyceride	
3	6	2.9
6	6	4.5
9	6	5.4
Lecithin	Monogalactosyl-diglyceride	
3	6	3.4
6	6	5.8
9	6	6.6
Lecithin	Digalactosyl-diglyceride	
3	6	3.2
6	6	4.8
9	6	5.7

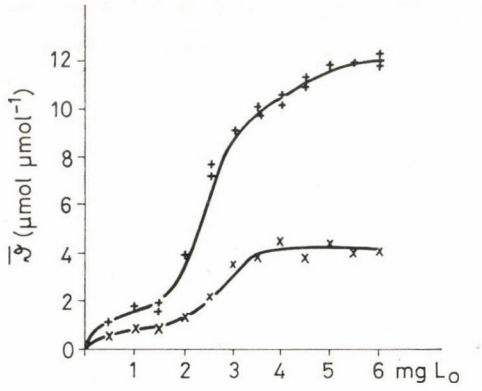

Fig. 10. Saturation curve of the model complexes of low molecular purothionin fraction reversibly modified at its arginyl side-chain with cyclohexandion and of phosphatidyl-ethanolamine. Final volume: 7.0 cm^3; protein content: 0.8 mg. Symbols: +—+ model experiment with the original protein and after removal of the blocking agent; ×—× experiment with chemically modified protein

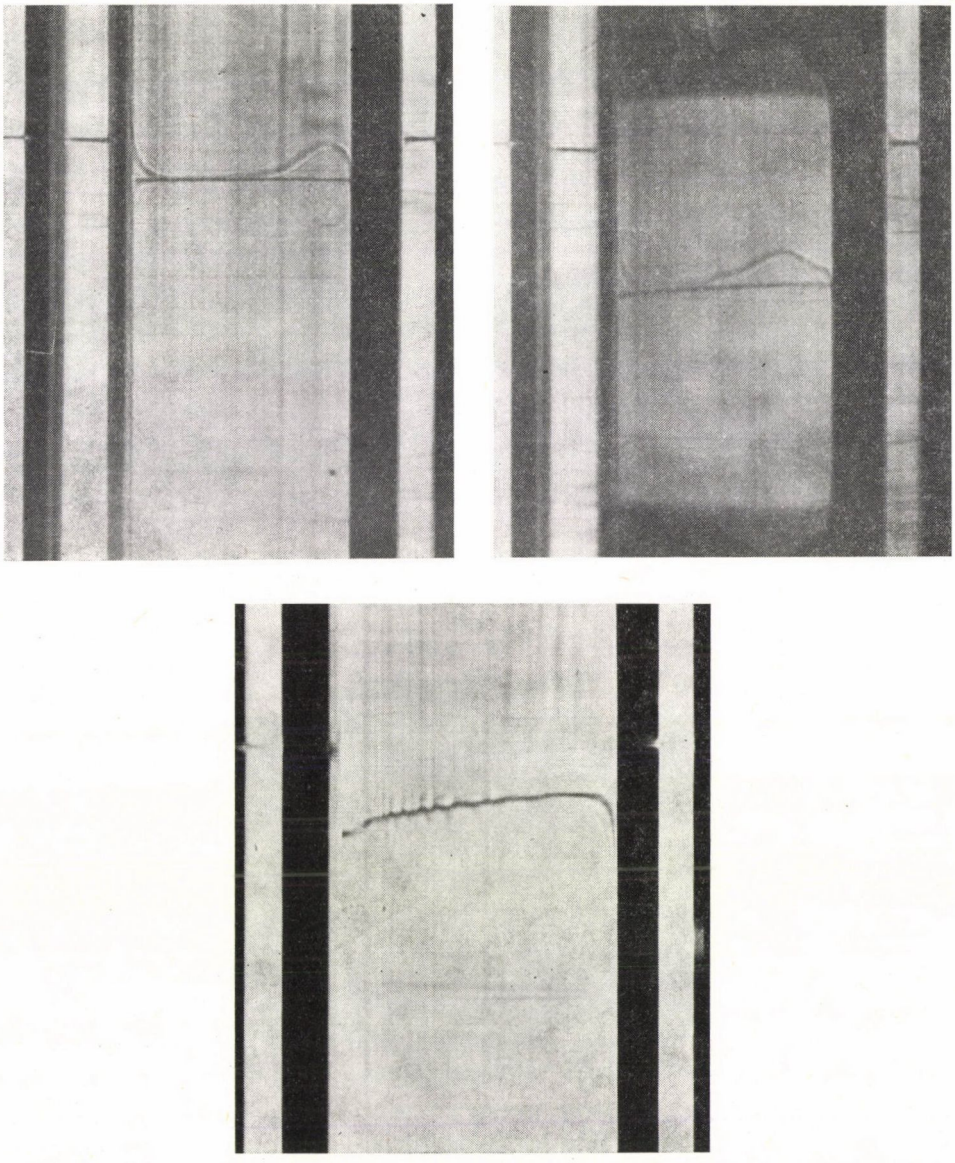

Fig. 11. Photos of the ultracentrifuge experiments with isolated purothionin and its model lipid complexes. *a:* Lipopurothionin 1.4% solution in chloroform; *b:* purothionin–lecithin (0.8 + 0.6 mg per 7 cm^3) precipitated complex 1% solution in chloroform; *c:* lipopurothionin 1.4% solution in chloroform. In experiments *a* and *b* rpm: 30 000, in *c* rpm: 50 000. Temperature in all three: 25 °C

In Fig. 12 the UV spectrum of the precipitated complex of model experiments carried out with purothionin, lecithin, lipopurothionin and the mixture of purothionin–lecithin, is illustrated. The two spectral characteristics of puro-

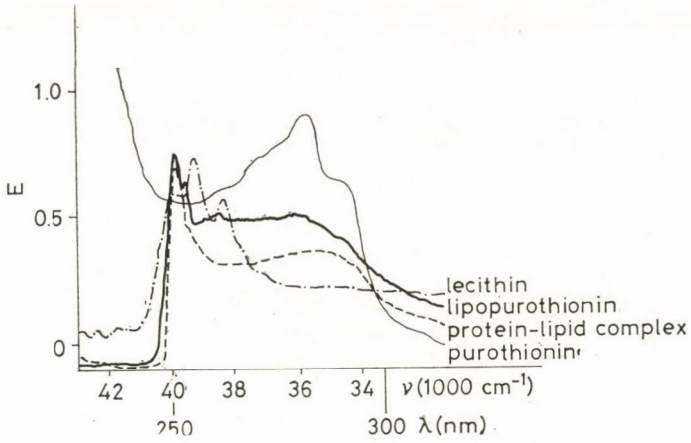

Fig. 12. UV spectrum of purothionin, lecithin, lipopurothionin and of the model complex solution according to Fig. 11/b

thionin, a maximum at 278 nm and a shoulder at 283 nm shifted in the same direction with both complexes, just like the peaks of the phosphatides at 252, 258 and 262 nm.

3. Conclusions

Data in the literature on the complexes formed by purothionin with lipids are rather contradictory. Whether the isolated proteolipids were formed *in vivo* and have perhaps a definite biological function, or are products of the petroleum ether extraction, is a question not clarified yet (BALLS & HALE, 1940; REDMAN & FISHER, 1968; FERNANDEZ DE CALEYA *et al.*, 1974; HOSENEY *et al.*, 1970, 1971; POMERANZ, 1976). It is possible that both types of complex occur.

It appears that the biological and biochemical properties of purothionin hitherto known, and the role it plays in the baking quality of wheat flour is connected, at least indirectly, with the lipid binding capacity of protein, whether the mechanism of toxicity to microorganisms or the processes occurring during the storage of wheat flour and affecting its baking quality, are examined (OKADA & YOSHIZUMI, 1973; PATEY *et al.*, 1976; RAO *et al.*, 1978). Thus, the formation of complexes *in vivo* is not at all excluded.

In this study, the problem was approached from a different angle. Independently from the location and function of complex formation, the cause of the lipid binding capacity of purothionin, inherent in its structure, the structure of the protein-lipid complex, its quantitative proportions and the nature of the interactions were sought for. The study of the lipid complexes

of purothionin permits, in the thorough knowledge of the structure of apoprotein, the investigation of the relationship between the protein structure (quality and quantity of amino acid side-chains, their sequential location) and the lipid binding capacity.

A review of the literature on thionins and on plant lipoproteins shows that, in contrast to those of membranes of microbial origin or of serum lipoproteins, a structural model aimed at could not yet be established. Therefore, by adapting the technique applied in the study of the above analogous structures, the authors sought for data explaining the composition of lipid complexes of purothionin obtained by different isolation methods and the stability and solubility characteristics of the complexes.

According to data of this study the complex formed by purothionin with lipids is unstable and from the aspect of molecular size heterogeneous. This was proven by the elution diagrams of the fractions obtained on *Sephadex* LH 20 column and also by the experiments with ultracentrifuge. In the protein part of the complex every fraction of purothionin, those of high and low molecular mass, may be found. The complete complex separated by gel filtration from the petroleum ether extract of wheat flour contains, in addition to the polar lipids as described in the literature (REDMAN & FISHER, 1968; HOSENEY *et al.*, 1970; HERNANDEZ-LUCAS *et al.*, 1977) a substantial amount of neutral lipids.

There is a significant difference in the gross fatty acid composition of the complete bound lipid fraction and that of the free lipid fraction in relation with purothionin.

It was found that the polar lipid fraction bound by stronger interaction to the proteins may not be considered homogeneous. Beside the greater part separated by *Folch* washing, there is a lipid fraction more stably bound and separable only by splitting with hydrochloric acid.

Based on the qualitative and quantitative analysis of the three bound lipid fractions, on the quantitative evaluation of the model complexes formed by purothionin and lipids and finally on the knowledge of the primary structure of purothionin, the structure of the complete complex may be described as follows:

The charged amino acid side-chains of purothionin are capable of binding, in salt-like interaction, polar lipids. The salt-like character of the interaction is supported by the strong pH dependence of the complex forming capacity as well as by the phenomenon well known from the related literature and related by the authors to purothionin substrate in accordance to which the presence of monovalent cations does not influence complex formation, while that of polyvalent cations exerts a strong inhibitory effect. The key role played by basic amino acids in complex formation is unambiguously proved by the model experiments after masking the proteins.

A special role is attributed to the sequential parts in the formation of salt-like interactions, where an amino acid side-chain of acidic character is followed by one of basic character, because these enter into relatively more stable binding with phospholipids having two ions forming with them double salt-like binding. This probability was derived from the knowledge of the sequence of α and β purothionin. The formation of this interaction was proven in model experiments with phosphatides having zwitter-ions in the first $\overline{\vartheta}$ step. The $\overline{\vartheta}$ value as measured in the low molecular mass fraction approximates the theoretical value of 2.33.[The fraction α contains two acidic basic sequence parts, (–10–11, 22–23–) while fraction β contains three, two as above and in addition a third at 41–42 sequence, the proportion α–β is 2 : 1.)

The LYS and ARG side-chains, having indifferent neighbours from the aspect of salt-like bond, interact with phosphatides by single salt-like bond. According to the $\overline{\vartheta}$ step of model experiments all the 12 basic side-chains of α and β fractions are capable of binding lipids. It is interesting to note that out of the theoretically expected $\overline{\vartheta}$ value of 6.33 in consequence of the blocking of ARG side-chains only a value around 4 was measured. This may be explained by the circumstance that because of the block effect of ARG 46, LYS 45 and 47 were poorly accessible.

In model experiments the complete purothionin showed a qualitative analogy with fractions of low molecular mass, interpretable exactly in the knowledge of their sequence. While to the fraction of low molecular mass α–β belongs a value of 2.33–12, to the complete purothionin of an average mass of 5000, belongs 1 pair of acidic basic side-chain and 7 basic amino acid side-chains.

In analysing the isolated complexes, the relatively stable complex known as lipopurothionin was found to contain a substantial amount of glycolipid and little sterolester. The model experiments have shown that glycolipids may be integrated into the complex only if a protein–phospholipid complex is already existing. The data as to the quantity of glycolipids suggest the formation of phospholipid–glycolipid bonds rather than protein–glycolipid bonds. Similarly, as can be concluded from the orientated location of phosphatides and from the lability of the complete complex, the complexing of neutral lipids is interpreted as phospholipid–neutral lipid interaction (not excluding the hydrophobic protein–lipid interrelations formed between hydrophobic amino acid side-chains and neutral lipids).

Figure 13 gives a schematic diagram of the hypothetic structure of the lipid complexes of purothionin fractions of low molecular mass, as derived from the results of measurements.

The conformation structure as shown in Fig. 13 originates from the work of MAK and JONES (1976). Based on the purothionin fractions (α_1, α_2, β) isolated from wheat flour and the relation of parts of sequences considered conservative

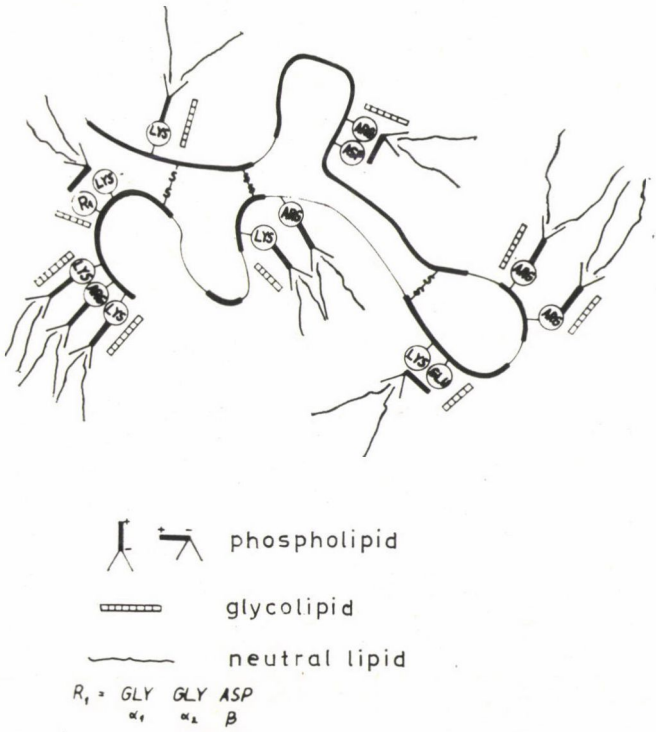

Fig. 13. Hypothetic structure of the purothionin–lipid complex

by comparison to viscotoxin, to lipid binding locations and on the study of purothionin analogs isolated from barley, rye and in accordance with the literature at first from oat (BÉKÉS, 1980), it is assumed that the family of thionins is a universally wide-spread protein-like compound of the plant world, biologically active, provided with a biologically important function of lipid binding capacity.

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Abstracts

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RELATIONSHIP BETWEEN THE ACTIVITIES OF SOME SERUM ENZYMES, PRE-SLAUGHTER STRESS AND OCCURRENCE OF PSE-MEAT IN SWINE

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The activities of the enzymes CPK, Ald, LDH, AP, GOT and GPT were determined in a total of 63, 200–210-day-old lowland swine of 105 kg weight, before delivery to the slaughterhouse as well as in blood samples taken at slaughtering. In the course of the *ante mortem* investigation the behaviour, respiration, the frequency of stool and urination and the colour of the skin were observed. The investigations into meat quality were extended to the values of pH_1 and pH_{24} as well as the sensory testing of *m. longissimus dorsi* and to the instrumental determination of the colour and the water retention capacity.

Enzyme activities were found to be higher in the three experimental groups at slaughtering than before transportation. The increase in activity was particularly marked with LDH and CPK.

The swine kept under closed conditions and slaughtered immediately after delivery at the slaughterhouse (group III, 25 hogs) showed the symptoms

of the undesirable stress state prior to slaughtering. The activities of CPK, Ald and LDH in the blood samples taken at slaughtering were highest in this group (522, 11.8 and 652 U l^{-1}, resp.). In 28% of the animals a grave, while in 56% a moderate PSE alteration of the meat was observed. The animals kept under closed conditions, but allowed to rest after transportation and before slaughtering for 1 day (group II, 22 swine) had in the blood sera taken at slaughtering CPK activities nearly as high (506 U l^{-1}) as the preceeding group, while the LDH activities were lower (486 U l^{-1}). The mean values of some of the laboratory parameters (pH, colour) related to meat quality, but especially the values of some individual animals indicated a mild PSE alteration. In groups II and III particularly high CPK and LDH values were obtained for the individuals the meat quality parameters of which were unfavourable, too. The swine allowed to move systematically and daily and allowed to rest for 1 day after transportation to the slaughterhouse and before slaughtering (group I, 16 swine) showed considerably lower activities of CPK and LDH (297 and 192 U l^{-1}, resp.) than the previous groups. The swine belonging to groups I and II were calm before slaughter and no significant difference could be established sensorically between the two groups as related to meat quality. In group I no values indicating PSE alteration were obtained. The activities of the enzymes AP, GOT and GPT were nearly identical in the three groups.

INVESTIGATION INTO THE OCCURRENCE OF ISOENYZMES IN THE CULTIVATED MUSHROOM

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Some isoenzymes of the cultivated mushroom (*Agaricus bisporus*) were repeatedly studied by the use of polyacrylamide gel electrophoresis in gels containing 7 per cent of acrylamide. With catechol and proline as substrates the *o*-diphenol oxidase yielded 3 broad bands in the extract of the pileus and 2 bands in the stipe. When tyrosine was used as substrate, only one band appeared in both extracts, but the band of the pileus was more broad and intensive. The oxidoreductases were investigated in the developed gels by incubating with suitable substrates and iodo-nitro-tetrazolium chloride. Comparing young and mature fruiting bodies it has been established that in the case of lactate dehydrogenase the pileus of young and mature mushrooms contains two or three more isoenzymes than the stipe, and the activity is higher in the mature ones.

The occurrence of mannitol dehydrogenase isoenzymes is also higher in the pileus than in the stipe. The number of dehydrogenase isoenzyme bands may change with the age of the fruiting bodies even if they are gathered in short periods at the same state of development.

CHANGES IN SOME ENZYME ACTIVITIES OF CEREALS DURING RIPENING

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In order to attain a better quality of the cereals to be processed, the enzymological investigations have been extended to the period of ripening. Researchers are on the look-out for correlations between the changes occurring during ripening and the properties of the final product (MARCHYLO *et al.*, 1976; SARGEANT & WALKER, 1978). The authors have investigated the amylase and peroxidase activities of the wheat varieties *Martonvásári 4* (Mv 4) and *Bezostaya 1* (B 1) from flowering till harvest.

The wheat samples investigated in the years 1978 and 1979 were obtained from an experiment carried out on small parcels. The wheat samples taken from the ears were first analyzed 4 to 5 days after flowering (June 5 and June 4 in the two consecutive years, resp.) and later at 7-day intervals. Seven days after harvest the measurements were repeated. The wheats were harvested in the first year on the 7th and 8th week, resp., in the second year on the 5th week, owing to the higher temperature of the air.

After comminution of the wheat the enzyme extract was prepared by extraction with distilled water. Measurement of amylase activity was carried out with the substrate *Phadebas Amylase Test*. For the determination of peroxidase activity hydrogen peroxide was used as acceptor and *o*-phenylene diamine as hydrogen donor (1 Unit = 10^{-3} Δ OD min^{-1}).

The amylase activity of the wheat Mv 4 decreased in the year 1978 from the 1st till the 5th week and in the year 1979 from the 1st till the 3rd week. During the further two weeks of ripening the amylase activity of the wheat in the ears increased. In the first year of the experiment the amylase activity of the wheat Mv 4 was 1 240 U g^{-1}.

In the first year of the experiment the peroxidase activity decreased till the 4th week (30 kU g^{-1}), afterwards the activity value increased till harvest. In the second year of the experiment the activity values increased from week to week up to the 4th week, while peroxidase activity decreased on the 5th week. The highest peroxidase activities observed during ripening

were 78 kU g^{-1} and 82 kU g^{-1}, resp., in the two years. The two enzymes investigated showed their highest activity values in the two years on the 7th and 4th week, resp., and these values were very similar in the two years.

In the case of the wheat B 1, amylase activity decreased in the year 1978 till the 6th week, thereafter it increased during the further two weeks of ripening. In the following year amylase activity decreased till the 5th week, *i.e.*, till harvest.

In the year 1978 peroxidase activity remained nearly at the same level till the 5th week of the ripening period (33–36 kU g^{-1}). From the 5th to the 7th week it increased to decrease on the 8th week, before harvest. In the year 1979 peroxidase activity increased weekly during the period of investigation, *i.e.*, till the 5th week. The highest peroxidase activities were 70 kU g^{-1} and 72 kU g^{-1} during ripening in the years 1978 and 1979, resp. In one year peroxidase activity was maximum on the 7th and in the other on the 5th week before harvest.

Peroxidase activity was highest in the period of full ripening. In the two consecutive years, in spite of the different weathers, the maximum values of peroxidase activity for a given variety were nearly identical.

In both years the amylase activity in the wheat Mv 4 was higher and the peroxidase activity was lower after harvest than in the wheat B 1.

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CHANGES IN AMYLASE AND PEROXIDASE ISOENZYME PATTERNS IN WHEAT DURING RIPENING

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A study has been carried out to investigate the amylase and peroxidase disc electrophoretic isoenzyme patterns of *Triticum aestivum* var. Martonvásári 4 from flowering till harvest.

Wheat samples harvested in 1979 were taken weekly after flowering. The samples were comminuted in distilled water. The disc electrophoretic systems of DAVIS (1964), REISFELD and co-workers (1962) were slightly

modified. Runs were carried out at pH 7.9 and pH 4.8, resp., from cathode to anode and with the electric field reversed. Protein loads corresponding to 20–80 mg of wheat (wet weight basis) were applied per gel. Electrophoresis was performed in the gel electrophoretic chamber of REANAL (Budapest) at room temperature, using 4 mA current per gel. Amylase isozymes were detected by immersing the gels into 1% soluble starch solution in 0.1 mol l^{-1}, pH 4.8 acetate buffer, at 50 °C for 4–5 min, then placing into a 0.4% KJ solution containing 0.04 g J_2. Peroxidase fractions were detected by dipping gels for 15 min into a solution containing 1 mmol l^{-1} *o*-dianisidine and 0.1% hydrogen peroxide in 0.2 mol l^{-1}, pH 4.8 acetate buffer.

Both enzymes were present in multiple forms. The amylase fractions proved anionic *at the alkaline* and cationic *at the acidic pH*. The number and activity of the amylase isozymes separated *at the alkaline pH* were lower than *at the acidic pH*. *The alkaline system* showed a fast moving amylase band present throughout ripening. A second band was detectable only at the first sampling. *In the acidic system* the number of amylase isozymes varied from 2–8 with changing intensities.

In the samples taken during ripening all peroxidase isoenzymes were positively charged *at the acidic pH*. Their number varied from 4–7 and amounted to 13 in the wheat analyzed after harvest.

In the alkaline system only anionic peroxidase fractions were present during the ripening period. A cationic fraction of very low mobility could be detected only occasionally. In the sample taken after harvest no anionic band could be revealed, while several distinctly coloured isoenzymes were found to migrate to the cathode.

In earlier studies only peroxidase isoenzymes positively charged *in an alkaline system* were found in wheat during storage (NÁDUDVARI-MÁRKUS *et al.*, 1979) while ripening wheat showed only anodic fractions. This is in agreement with the present findings. However, in the earlier year a faint anionic band was still present in the harvested sample. HONOLD and STAHMANN (1968) reported on the presence, in a similar electrophoretic system, of cathodic peroxidase isozymes, however, mainly in spring wheats.

The number of anionic amylase isozymes detected *in the alkaline* electrophoretic system was considerably lower as compared to the results of KRUGER (1972) obtained with hard red spring wheats in a similar system.

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EFFECT OF EMULSIFIERS ON THE ACTIVITIES OF STARCH-HYDROLYZING ENZYMES IN THE BAKING INDUSTRY

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Three types of emulsifiers – monoglycerides (MG), diacetyltartaric acid esters of monoglycerides (DATA) and lactylates (CSL) – are approved for use in bakery products in Hungary.

The effect of emulsifiers on starch gelatinization, on the activity of starch-hydrolyzing enzymes as well as on starch retrogradation were studied with the amylo-viscograph at our institute. Commercial flours (BL 55, BL 80, BL 112) and meals prepared from other known wheat varieties were used.

Starch damage of flours was examined by a spectrophotometric method (blue colour reaction of iodine) and the protease activity of flours measured spectrophotometrically on the synthetic substrate L-BAPA. Amylase activity was determined by a chemical method (according to the *Phadebas test* by *Barnes*) and as usual in industrial practice, by the *Hagberg* falling number and with the amylo-viscograph.

The value obtained for amylase activity by the chemical method was independent of protease activity or starch damage. The value of the *Hagberg* falling number is influenced by the amylase activity and protease activity, and the third peak of the amylo-viscogram is influenced by amylase activity and starch damage.

The first peak of the amylo-viscogram is related to starch gelatinization and to the activities of the amylolytic enzymes. The third peak is related to ageing. Both peak heights decrease with increasing amylase activity. Characteristic temperature values of gelatinization are reduced in parallel to a rise in amylase activity values. The temperature range of gelatinization becomes narrower through the action of emulsifiers.

The staling rate of the end-product is influenced by amylase activity. With an increase in enzyme activity the staling rate is lowered. Emulsifiers, too, affect the staling rate. The strongest retarding effect on the firming rate was found to be achieved by the addition of lactylates.

Experimental data proved the use of anti-staling agents solely to be insufficient to achieve the necessary retarding effect on the firming rate. The amylolytic state of flour must be adjusted to an optimum level (*Hagberg* falling number around 250), as, if starch hydrolysis is not complete, the anti-staling agent cannot exert its influence in a proper way.

INVESTIGATIONS INTO THE CHANGES OF SOME SUGAR BEET ENZYMES DURING STORAGE

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The changes occurring in sugar beets during storage are closely related to the general biochemical state and composition of the sugar beet plant. The changes taking place during ripening cannot be strictly separated from each other, however, some enzymes of primary importance can be selected that act at relatively distant points of the metabolic chain. One of the main reasons of sugar losses is respiration, one of the possible limiting factors of which is the value of invertase activity. From the technological aspect the solid matrix formed by the pectic substances is important as well. First of all, pectolytic enzymes are responsible for the decrease in firmness. The browning of the beet extract is a sensitive factor in technology, the enzyme polyphenol oxidase being responsible for the process.

The activities of invertase and polyphenol oxidase (PPO) have been determined with the automatic analyzer *Contiflo*. Invertase activity was assessed by measuring the difference, in inverted sugar content, of the original sample and the sample inverted at 50 °C for 5 h, applying a method based on the reduction of ferricyanide. PPO activity was characterized by the change in optical density occurring during 1 min at 25 °C, in citrate–phosphate buffer with catechol as substrate.

The activity of pectolytic enzymes was determined in an *Ostwald–Canon–Fenske*-type viscometer; the decrease in viscosity of a pectin solution was measured at 35 °C.

Sugar beet samples of the same variety treated by combining two different levels of fertilizers and irrigation have been investigated. In early harvested samples invert sugar content and invertase activity increase at the beginning very rapidly with storage time, while in later harvested, riper samples both variables show a steep increase towards the end of storage. The increase in activity of the irrigated samples treated with higher doses of fer-

tilizers is higher than the average. In early harvested samples pectinase activity is not considerable and does not increase significantly during storage. In later harvested samples activity shows a slow increase during storage. A considerable increase in PPO activity can be observed in early harvested beets, especially with beets grown at low doses of fertilizer and not irrigated. In samples from the second harvest PPO activity decreases depending on the conditions of growth. Decrease is lowest in the irrigated samples treated with higher levels of fertilizer and highest in beets not irrigated and treated at low doses of fertilizer. Further investigations might permit of predicting changes occurring during ripening.

INFLUENCE OF LOCATION AND YEAR OF HARVEST ON SOME OXIDATIVE ENZYME SYSTEMS OF APRICOT CULTIVARS

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Mass-related polyphenol oxidase (PPO) and peroxidase (POD) activities, initial rate of enzymatic browning (BA) and *o*-dihydroxy phenol content (ODP) of 10 apricot cultivars from two locations (C = Central Hungary, W = Western Hungary) have been determined, during 4 consecutive years, by methods published earlier (Gajzágó & Vámos, 1975; Mihályi & Vámos, 1976).

The characteristics studied varied considerably with cultivar, location and year. Variations were more pronounced in ODP and PPO activity than in BA. The ratios of the highest and lowest values established were, in the above order, 46, 17 and 6.

Cultivars from location W had higher or similar BA and higher PPO and POD activities than the respective cultivars from location C. ODP and POD activity seem to be cultivar-dependent.

In contrast to earlier findings obtained with apples (Vámos *et al.*, 1976; Vámos *et al.*, 1977), BA in apricots was found to be primarily dependent on endogenous substrate content. However, the population investigated did not prove homogeneous in this respect: a distinction could be made on the basis of the PPO : ODP ratio. For the 13 lots in which the ratio exceeded the numerical value of 0.35, the relationship between BA, PPO activity and ODP as determined by multiple linear regression analysis, could be described by the equation $BA = 0.432 + 0.0373\ ODP + 0.0116\ PPO$; $R^2 = 0.7298$; $R = 0.8542$; $P = 99.9\%$. For the 9 lots in which the value of the ratio was below 0.35, the relationship fitted the equation $BA = 0.98 + 0.0044\ ODP + 0.0244\ PPO$; $R^2 = 0.8781$; $R = 0.9371$; $P = 99.9\%$.

The quotients of the partial regression coefficients showed the influence of ODP on BA, in the former population, to be 6-fold and in the latter 2.6-fold that of PPO.

The striking heterogeneity of the lots might, among others, be connected to differences in maturity of these quickly ripening fruits.

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INVESTIGATIONS INTO THE HEAT RESISTANCE OF PEROXIDASE

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The unfavourable changes in flavour, colour and consistency occurring during storage of horticultural products are partly attributed to the action of the enzyme peroxidase, therefore heat denaturation of the enzyme is an important step in the technology of food preservation. Owing to its high heat resistance, kohlrabi peroxidase has been selected as model substance for investigations.

The water extract of kohlrabi peroxidase was treated at 65, 70, 75, 80, 90 and 95 °C for 0, 1, 3, 5, 10 and 15 min, resp., then allowed to stand at room temperature for one day. According to preliminary experiments enzyme regeneration occurred, during this time, to its full extent. Heat treatments were performed under conditions ensuring negligible heating and cooling periods. Peroxidase activity was measured immediately after heat treatment and after full enzyme regeneration.

Activity values measured after heat treatments showed inactivation to be biphasic at all temperatures and within all time lapses applied. After 0, 1, 3 and 5 min of heat treatment at 65, 70 and 75 °C, resp., activities increased as related to the respective values of untreated samples. This might be account-

ed for by the formation of more active conformations formed by rearrangement during heat treatment or by the heat denaturation of an inhibitor originally present. Heat treatments of 10 and 15 min, resp., decreased enzyme activity at rates increasing with temperature. Within this interval inactivation corresponds to a monophasic first order reaction and represents the inactivation of the heat labile isoenzymes. The heat stable enzymes were not inactivated even by 15-min heat treatments. At 80, 90 and 95 °C heat labile isoenzymes were inactivated instantaneously. Heat stable isoenzymes were inactivated upon further heat treatment according to first order kinetics.

Investigations into the regeneration of the partially inactivated enzyme as a function of the duration and temperature of heat treatment showed maximum regeneration taking place in samples treated at 70 °C. Both at lower and higher temperatures regeneration was less, above 70 °C regeneration decreased with increasing temperature and time of heat treatment.

ESTIMATION OF THE MALATE DEHYDROGENASE ACTIVITY OF SOME VEGETABLES AND FRUITS

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The authors measured the malate dehydrogenase activity in some vegetables and fruits. The determination was carried out with the method of *Bergmeyer–Bernt*, according to the following reaction

$$\text{L(-)-malic acid} + \text{NAD}^+ \xrightleftharpoons{\text{malate dehydrogenase}} \text{oxalacetate} + \text{NADH} + \text{H}^+$$

utilizing the reaction taking place in the direction of the lower arrow (pH = 7.4).

Samples of the horticultural products ripe for consumption and for processing, resp., were selected for the investigations. Some informatory results are given in the Table.

In the course of the experiments it was established that the malate dehydrogenase activity of tomatoes decreases considerably during ripening. A decrease in enzyme activity was observed also during the storage of pears and cauliflower. The difference between the results obtained with cauliflower was great, depending on the place of sampling (stalk, flower).

The experiments will be continued in order to study the deviation between cultivars and years as well as the changes in malate dehydrogenase activity occurring during ripening and storage.

Malate dehydrogenase activities of some vegetables and fruits

Name	Activity (μmole g^{-1})
Tomato	146
Cultivated mushroom	327
Cucumber	1 850
Vegetable marrow	4 172
Paprika (tomato shape)	5 528
Onion	6 740
Onion, sprouted	7 708
Cauliflower (early)	8 700
Cauliflower (late)	13 175
Apple *(Starking)*	95
Pear *(Serres Olivér)*	140

PRODUCTION OF AND INVESTIGATIONS INTO IMMOBILIZED GLUCOAMYLASE

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In manufacturing starch degradation products saccharification with glucoamylase is the process most often used. The glucose formed by saccharification can be used as crystalline dextrose, "total sugar", glucose–fructose syrup, sorbitol to manufacture products of the fermentation industry, *etc.* The process is carried out today with soluble glucoamylase in a batch process, maintaining the reaction mixture in general at 60 °C for a long period. The application of an immobilized enzyme would mean in this case the realization of the continuous automated saccharification and economical advantages in saving heat and mechanical energy.

Numerous methods have been elaborated (Solomon, 1978) for immobilizing glucoamylase, however, the introduction of the process into the industry is still to be waited for. One of the main reasons of this is that the supports used up till now are expensive or not suitable for industrial application. The authors have studied the applicability of bead-form cellulose amine and DEAE-cellulose. The immobilization of the enzyme has been carried out, in the case of 2-amino-ethyl-O-cellulose, with glutaraldehyde and *p*-benzoquinone and, in the case of DEAE-cellulose, with *p*-benzoquinone. The results of immobilization are summarized in Tables 1 and 2.

From Table 1 it can be established that the enzyme has been bound mainly on the surface of the particles. It is true that the content of amino groups is also strongly surface-depending. The stabilities and half-lives of both immobilized enzymes are much better than those of the soluble enzyme (half-life at 50 °C is about 48 days, at 60 °C about 14 days), the optimum pH is decreased from 4.2 to 3.5.

Table 1

Immobilization of glucoamylase to 2-amino-ethyl-O-cellulose

No. of sample	Particle diameter (μm)	$-NH_2$ content (mole kg^{-1})	Bed volume (l kg^{-1})	Immobilized activity (μmole G min^{-1} g^{-1} support)	
				glutar-aldehyde	benzoquinone
				support	
				0.4 g g^{-1}	0.5 g g^{-1}
1	30	0.60	3.3	190	290
2	131	0.37	2.7	105	200
3	213	0.24	2.5	60	120

Table 2

Immobilization of glucoamylase to DEAE-cellulose
(particle diameter: 160 μm in the dry state)

No. of sample	DEAE content (mole kg^{-1})	Bed volume (l kg^{-1})	Immobilized activity (μmole G min^{-1} g^{-1} support)
1	0.6	3.8	79
2	0.9	9.4	510
3	1.1	14.0	330

From Table 2 it can be established that here the enzyme has been bound also in the pores, the best results have been achieved with the support swelling to 10 liter per kg. The respective product has been tested in reactors of different types and the parameters of application yielding optimum utilization have been established. The most important factor is the "stationary diffusion boundary layer" formed around the particles containing the immobilized enzyme. The diffusion-inhibiting effect of this layer has to be taken into account in developing the industrial technology (Holló *et al.*, 1978).

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INVESTIGATIONS INTO THE REACTOR KINETICS OF IMMOBILIZED GLUCOSE ISOMERASE

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Glucose isomerase (GI) is, for the time being, the only immobilized enzyme preparation applied on an industrial scale in the food industry.

A fundamental requirement of the economical industrial technology is an inexpensive immobilized enzyme. This can be achieved, in the first place, by immobilizing cells.

In their experiments the authors applied *Streptomyces* cells produced by the Research Institute of the Pharmaceutical Industry (560 GIU g^{-1}), the supports 2-amino-ethyl-O-cellulose (cellulose amine) and 2-amino-ethyl-O-starch (starch amine), gelatine as additive and immobilization with glutaraldehyde.

Some characteristic parameters of the immobilized enzymes prepared on the basis of optimization experiments have been summarized in Table 1 as compared to similar parameters of two commercial preparations.

It was established that starch amine is far superior to cellulose amine, while gelatine improves the mechanical and enzyme stability properties of the product to a considerable extent.

In order to compare the individual preparations and to establish their industrial applicability, beside the characteristics generally investigated, the changes in K_m values, outer and inner diffusion inhibitions and pore size have been studied as well. The dependence on temperature and particle size of the K_m values of the preparation Sweetenzyme E as measured in a column reactor are shown in Table 2.

Table 1

Some characteristic parameters of the immobilized enzymes prepared on the basis of optimization experiments

Immobilized GI (GIU/g)	Activity (GIU g^{-1})	Water binding capacity (%)	K_m (mole l^{-1})
Sweetenzyme E (NOVO)	200	384	0.85
Miles enzyme	348	371	1.08
20% starch–NH_2	295	310	3.10
20% starch–NH_2 + 20% gelatine	369	353	0.78
20% cellulose–NH_2	186	290	—
20% cellulose–NH_2 + 20% gelatine	251	290	—

Table 2

The dependence on temperature and particle size of the K_m values of the preparation Sweetenzyme E as measured in a column reactor

Average particle size (mm)	K_m value (mole l^{-1})				
	45 °C	50 °C	55 °C	60 °C	65 °C
0.22	0.29	0.49	0.71	1.06	1.37
0.39	0.31	0.53	0.63	1.09	1.42
0.64	0.30	0.55	0.59	1.02	—
0.82	0.34	—	0.58	—	1.39

The outer diffusion inhibition has been calculated from the K_m values by graphical determination of the material transport constants k_m (PITCHER, 1975), while the inner diffusion inhibition has been determined by an indirect method, calculating the efficiency factor "η" (BUNTING & LAIDLER, 1972). The dependence of the values of k_m and η on the particle size is given in Table 3.

From the results it can be seen that the outer diffusion depends but to a small extent on particle size and that the decrease in particle size results in a decrease in material transport. Practically the values η do not depend either on temperature or on particle size, indicating that there is no inner diffusion inhibition. This latter fact can be borne out also by the investigations into pore size. According to our measurements a molecule of the molecular mass of about 1000 is able to enter the pores of the immobilized enzyme, glucose being a far smaller molecule.

Table 3

Dependence of the values of k_m on the particle size

Average particle size (mm)	k_m (cm min^{-1})	Average η
0.22	$4.3 \cdot 10^{-2}$	0.78
0.39	$5.7 \cdot 10^{-2}$	0.76
0.64	$6.6 \cdot 10^{-2}$	0.76
0.82	$6.9 \cdot 10^{-2}$	0.77

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PREPARATION OF IMMOBILIZED ENZYMES FOR THE PURPOSES OF THE FOOD INDUSTRY

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The immobilization of *Bacillus subtilis* alpha-amylase and *Aspergillus awamori* SA 586 glucoamylase by adsorption as well as that of various proteases (trypsin, Alcalase, alpha-chymotrypsin, Pronase) by covalent binding has been investigated.

Immobilization by adsorption was carried out with a phenol-formaldehyde polycondenzation resin, immobilization by covalent binding with a metacrylic acid-styrol-divinylbenzene copolymer containing maleic acid anhydride as chemically active agent. The preparation of the polymers was performed according to two Hungarian patents.

The applicability of our immobilized enzyme preparations in starch and protein degradation processes was studied.

Our results can be summarized as follows:

1. 3×10^4 SKB units of alpha-amylase adsorbed to 15 cm^3 of phenol-formaldehyde resin hydrolysed a 2% potato starch solution previously digested by autoclaving in 16 hours at pH 5 and 37 °C to 95–98%. Degradation was followed with the iodine reaction. (Without autoclaving, the given system degraded potato starch but to 25%.) Degradation took place in a fluid resin reactor. After eight repetitions of the process the enzyme—resin complex did not show any decrease in activity.

2. Our system described in point 1 degraded 60 g soluble starch in a 10% solution in 1 h at 50 °C to 80–90%.

3. The solution of 20 g potato starch prehydrolyzed with the aid of the alpha amylase—resin complex could be hydrolyzed to glucose with an output of 90–95% applying 98.0 AGU glucoamylase adsorbed to 15 cm^3 of phenol-formaldehyde resin at pH 4.5 and 50 °C. Hydrolysis in the fluid resin reactor could be reproduced eight times giving the same results.

4. When immobilizing proteolytic enzymes with copolymers containing maleic acid anhydride, the activity of the covalently bound enzyme as measured on casein substrate decreases to 50–80%. A detailed study of the Pronase enzyme from *Streptomyces fradiae* showed that the decrease in activity could be noticed only with casein as substrate, when applying a low molecular mass substrate the total enzyme activity could be recovered.

APPLICATION OF ENZYME PREPARATIONS IN PREPARING RED WINE BY THERMAL PROCESSING

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Red wine processing by thermal treatment is spreading throughout the world and thus also in Hungary and makes processing under industrial conditions possible. The chemical composition and quality of red wine can be influenced by the temperature of heating and the duration of maceration. However, when heating to temperatures above 55 °C, sedimentation and handling of the wines thus obtained becomes difficult. Enzymatic treatment of the must containing grapes or the must or wine might promote and accelerate the process of self-purification, the wines can be easily clarified and filtered.

The present paper deals with the time of treatment (must containing grapes, must or wine), the colouring matter-solubilizing effect of the individual enzyme preparations, the values of and changes in the characteristic chemical constituents of red wines, the comparison of the individual preparations and describes the results of the laboratory and industrial scale experiments.

TREATMENT OF YEAST BIOMASS BY ENZYMATIC HYDROLYSIS

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Yeast slurry, the by-product of brewing is a valuable feed protein source for its biological protein value (PER = 70% with reference to casein) and high vitamin B content.

The drum-dried product is unsuitable for direct human consumption because of its dark colour and bitter taste. A de-bittered and light coloured yeast powder might be produced by washing the yeast slurry with 4-fold volume of water (5 repetitions) and a careful spray-drying. This process of washing, settling and drying consumes large amounts of water, energy and time. To eliminate hop substances, yeast slurry equivalent to 1 kg of solid content must be diluted to 100 l, then separated and these operations have to be repeated 5 times. Ten liter of water have to be evaporated to produce 1 kg of dry yeast. The procedure gives a yeast powder of very good quality, neutral taste and colour significantly lighter than that of the original material.

Experiments were carried out to produce a light-coloured yeast powder of neutral taste using enzymatic treatment of the crude slurry to remove taste and colour substances.

We tried to achieve this goal using proteolytic enzymes and proteolytic + cell wall lytic enzymes, resp. Several proteolytic enzymes were investigated (papain, pepsin, alcalase, pronase, ficin, molsin, α-chymotrypsin). Cell wall destruction was performed with the digestive enzyme of *Helix pomatia*.

We expected not only the removal of undesirable taste and colour substances but an increase in the degree of protein hydrolysis (αH), too, resulting in better digestibility.

The efficiency of lytic enzymes depends highly on the age of yeast cells. The brewery yeast slurry contains aged cells. In spite of this fact the snail enzyme was effective on freeze-dried yeast samples and fresh yeast slurry as well.

Both the results of colour determination (CIE-LAB system) and the number of proteolytic splits (αH) were better in case of cell wall lytic pretreatment than by proteolytic treatment only.

The adopted procedure with combined enzymatic treatment gives by a single washing—separation step similarly good results in decolourizing as the control process. The product has a neutral taste and the αH hydrolysis degree reaches 80% in 4 hours, the value which can be reached, even after repeated proteolysis with protein isolates only in 48 hours.

INVESTIGATION INTO THE MECHANISM OF THE PLASTEIN REACTION

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The plastein reaction allows the incorporation of limiting essential amino acids and thereby improves protein nutritional quality.

In our laboratory, L-methionine methyl ester was incorporated into the appropriate hydrolysates of casein and soybean protein isolates during the resynthesis reaction. Incorporation of methionine and reaction mechanism were studied as follows:

It was assumed that if L-methionine methyl ester was incorporated into the protein chain by covalent bonds, formation of an equivalent amount of methanol could be expected.

Therefore methanol content of the supernatant of products of the plastein reaction was extracted by steam distillation and determined by gas chromatography.

Investigation of precipitates formed in plastein reactions was carried out after dialysis. Amino acid content and change in the methionine portion were controlled by thin-layer ion exchange chromatography as well as by amino acid analysis.

A significant increase in methionine content of plasteins was found owing to incorporation of L-methionine methyl ester during the enzymatic resynthesis process. Our experimental results indicate the formation of a covalent linkage of methionine in plasteins.

INCORPORATION OF METHIONINE INTO GLUTEN PROTEIN BY A ONE-STEP PLASTEIN REACTION

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In order to increase the methionine content of gluten a recent variant of the plastein reaction, the one-step procedure was applied. While in the usual plastein reaction the incorporation of amino acids in the ester form includes two enzymatic processes, *i.e.* protein hydrolysis and resynthesis, in the one-step plastein reaction the incorporation of the methionine ethyl ester takes place at a high substrate concentration by papain treatment. In order to establish optimum reaction conditions the experiment was planned applying the orthogonal latin square scheme. In our experimental scheme 4 factors were varied at 4 levels and the effects were calculated. The factors varied were as follows: gluten concentration, papain : gluten ratio, gluten : methionine ratio as well as the concentration of cysteine.

KINETIC INVESTIGATION OF PECTIN ESTERASE ACTIVITY

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Reports dealing with pectin esterase (PE) can be found in the scientific literature for three decades (Phaff, 1947; McColloch & Kertesz, 1948). PE can be found in many fruits and vegetables and it is synthesized by many microorganisms. In the course of its action in a pectolytic enzyme complex pectin esterase hydrolyzes the methyl ester groups of the pectin molecule making it accessible to the depolymerizing polygalacturonases.

Pectin esterases, mainly of plant origin, are used for the production of low-methoxy pectins, which are important gelling agents in the canning and

confectionery industry. Plant PE-s act according to a non-random mechanism. Japanese authors (Ishii *et al.*, 1979) have recently found that the action of a fungal PE is of random mechanism. According to this result, low-methoxy pectin of high gel forming capacity could be produced from high-methoxy pectin with the use of PE of fungal origin.

In the course of our work, the PE component of the pectolytic enzyme complex of an *Aspergillus niger* strain was investigated. The enzyme was a pectin lyase type enzyme complex, precipitated from the culture filtrate by methanol.

PE activity was measured by determining the quality of hydrolyzed milliequivalent ester bonds by NaOH titration.

Factors promoting and/or retarding the enzyme action were determined.

The effect of temperature (20–60 °C) was investigated and 30 °C proved to be optimum. PE activity measured at 30 °C did not differ significantly from that at 40 °C, but was very highly significantly higher than that measured at the other temperatures applied.

pH 4.5 was found best for PE action, which was highly significantly higher than at pH 5.0 and very highly significantly higher than at the other pH values.

When the effect of the enzyme concentration was tested, PE activity increased at a linear rate with the increase in enzyme concentration in the range of 0.06–0.6 mg cm^{-3} during incubation times of 15 and 30 min, while longer incubation periods resulted in a linear response between 0.06 and 0.25 mg cm^{-3}.

With the increase in the concentration of the substrate (*Pomosin* pectin, degree of esterification: 70%; Pomosin-Werke GmbH, FRG) from 1.5 to 10 mg cm^{-3}, PE activity increased from 0.262 to 0.438 mval cm^{-3}.

The values of V_{max} and K_m calculated from the *Lineweaver–Burk* plots proved to be 0.477 cm^{-3} and 1.445 mg cm^{-3}, resp.

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KINETIC INVESTIGATION OF PECTIN LYASE ACTIVITY

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In the course of the decomposition of pectin by pectin lyase, the enzyme attacks the pectin chain without water uptake through a transeliminative reaction, resulting in an end product of unsaturated galacturonic acids or their oligomers.

Pectin lyase (PL) was isolated from different commercial pectic enzyme preparations (Amado, 1970; Voragen, 1972). Pectin lyase of *Aspergillus japonicus* and *Aspergillus soyae* clarified apple juices to a great extent and had also a rather good macerating effect (Ishii & Yokotsuka, 1971a, b; 1972; Ishii, 1976).

In the course of our work, pectin lyase activity of the pectolytic enzyme complex of an *Aspergillus niger* strain was increased by NaN_3 mutagenic treatment (Zetelaki-Horváth *et al.*, 1979) and by cultivation conditions. The enzyme was precipitated from the culture filtrate with methanol and optimal parameters of the activity of PL were investigated.

(*Pomosin* pectin [Pomosin-Werke GmbH, GFR] of 70% degree of esterification was used as substrate. PL activity was determined from the absorption of double bonds at 235 nm and calculated using a molar absorption coefficient of 5 500.)

Of the four temperatures investigated (20, 30, 40 and 50 °C) 40 °C proved to be the best (at a very high level of significance) for the PL activity.

The effect of pH on the activity of PL was tested in the range of pH 3.5–7.0 (at intervals of 0.5 pH). At pH 6.0 PL activity was very highly significantly higher than those obtained at the other pH values.

The effect of enzyme concentration on the activity of PL was investigated during a 3-h incubation period (15, 30, 60, 90, 120 and 180 min). With the increase in enzyme concentration from 0.01 to 0.2 mg cm^{-3}, PL activity increased linearly during the 15 and 30 min incubations. When the incubation period was longer than 30 min, the linear phase ended at an enzyme concentration of 0.1 mg cm^{-3}.

The effect of substrate concentration on PL activity was investigated in the range of 1.0 to 20 mg cm^{-3} after incubation periods of 15, 30, 45, 60, 70 and 90 min, resp. PL activity increased at a linear rate with the increase of the substrate concentration from 5 to 10 mg cm^{-3}. When PL activity was investigated as a function of the time of incubation in reaction mixtures of different substrate concentrations, PL activity increased to a great extent with the increase of the substrate concentration.

The rate of the enzyme reaction at various substrate concentrations, and the kinetic constants from the *Linewaver–Burk* plots were determined by regression analysis. The kinetic constants found were the following:

$$V_{max} = 0.169 \text{ min}^{-1} \text{ and } K_m = 32.96 \text{ mg cm}^{-3}.$$

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COLOUR TESTING OF TOMATO JUICES FOLLOWING ENDO-POLYGALACTURONASE TREATMENT

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For several years now our department has been carrying out investigations related to the endo-PG treatment of tomatoes in order to increase the condensation level of the machine harvested product. The drying of the extremely hard and stout varieties of high fibre content confronts the canning industry with considerable difficulties.

To decrease the fibre content, two methods of enzyme treatment – cold and hot – have been elaborated. By enzyme treatment the rough fibres not containing coloured substances are decomposed too, and remain in the product. This raises the question whether the enzyme treatment method affects the colour of tomato purée.

To answer this we carried out tomato colorimetry experiments. For objective colorimetry we used the instruments *Momcolor*, widely applied in

Hungary, and *Tomacolor*, a green—red quotientmeter, developed especially for the purpose of tomato colorimetry.

We worked with the following four different enzyme treatment parameters:

- control (no enzyme added),
- 0.05% endo-PG concentration,
- 0.1% endo-PG concentration,
- 0.05% endo-PG + 0.01% cellulase concentration.

The incubation took 30 minutes in a shaker of 330 rpm.

The results of the measurements were evaluated by mathematical statistical methods.

No significant differences were found between the samples either in comparison with the control or with each other. The results of the two instrumental measurements show a linear correlation.

We further investigated the refractive index and viscosity values as well as the juice yield of the tomato samples. The viscosity values showed a straight decrease while the refractive index and juice yield showed a slight increase as a function of enzyme treatment.

ENZYMATIC REDUCTION OF THE LACTOSE CONTENT OF MILK

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In Hungary healthier nutrition requires to extend milk consumption to as many people as possible. This objective is often hindered by the lactose intolerance of the organism. This deficiency occurs in the countries of the globe to different extent, in Europe it may affect about 15–20% of the population. The explanation of the phenomenon is that in the mucous membrane of the small intestine the formation of the enzyme lactase is reduced, thus the disaccharide present in milk, *i.e.* lactose, does not split to monosaccharides, and can not be resorbed by the organism in the form of disaccharide. Consequently, milk consumption is followed by flatulence, diarrhoea and general indisposition.

This undesirable phenomenon can be eliminated, if milk-sensitive persons consume milk in which 60–80% of the lactose content has been previously hydrolyzed by the enzyme lactase.

In our experiments the liquid enzyme preparation *Maxilact* LX 5000 (Gist Brocades N.V., Delft, Holland) was used. In laboratory experiments the quantity of the enzyme necessary for the treatment of milk and condensed milk of 40% solids content, resp., has been established to achieve hydrolysis of 60–80% of the lactose content of milk in 20 h at 5 °C and that of condensed

milk in 2–3 h at 35 °C. On the basis of the results 200 l of consumers' milk of reduced lactose content and 350 kg of dehydrated milk of reduced lactose content were manufactured in industrial scale experiments using the enzyme preparation.

Production of consumers' milk of reduced lactose content

For the treatment of consumers' milk 1 g of enzyme was applied per liter. Enzyme treatment brought about the splitting of 94% of the lactose content of milk to glucose and galactose. The milk was packed into 1/2 l polyethylene bags and kept for 48 h (guaranteed shelf life) in the refrigerator without quality deterioration. The milk tasted slightly sweet. Enzyme treatment increases the price of the milk by 80%.

Production of dehydrated milk of reduced lactose content

One thousand l condensed skim milk were treated with 80 mg% enzyme for 3 h at 35 °C, then the condensed milk was spray-dried in an *Anhydro* apparatus at a 160 °C air input and a 85 °C air output. In the milk powder 58% lactose was split. The powdered as well as the rehydrated milks were slightly sweet, the sensory panel found their taste superior to that of the powdered and rehydrated milks, resp., prepared without enzyme treatment. The enzyme-treated dehydrated milk cannot be distinguished by colour from the control sample. Enzyme treatment increases the price of dehydrated milk by 20%.

Preparation of ice cream from powdered milk of reduced lactose content

Ice cream powder and therefrom ice cream was prepared applying dehydrated milk of reduced lactose content as obtained on an industrial scale. The ice cream was compared to a similar product prepared with normal dehydrated milk as control. It was established that the powdered ice cream prepared with dehydrated milk of reduced lactose content requires 12% less sucrose and the ice cream prepared therefrom is creamier than the control ice cream.

PRODUCTION OF A RENNIN-LIKE ENZYME IN SUBMERGED CULTURE OF ENDOTHIA PARASITICA: A KINETIC STUDY ON GROWTH AND ENZYME PRODUCTION

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It is a well known effort in microbiological enzymology to substitute the enzyme calf rennet used for cheese making by enzymes of similar action obtained from plants or microorganisms. This possibility has a great impor-

tance from the point of view of economy. Therefore, during the last years the basis of the production of a rennin-like enzyme in submerged culture of *Endothia parasitica* has been elaborated.

In order to increase the milk clotting activity in the fermentation broth the changes of the fermentation parameters – cell growth, milk-clotting activity, proteolytic activity, pH, decolourization time of the resazurin solution – and the relationship between cell growth and milk clotting enzyme production were investigated during cultivation.

The mycelium content started to increase 20 hours after inoculation. The maximum of dry mycelium content appeared at the age of 50 hours.

The milk clotting activity of the fermentation broth increased together with the mycelium concentration. It reached the maximum value between 45 and 52 hours, then decreased rapidly.

The change in proteolytic activity seemed to be closely associated with the change in milk clotting activity during the cultivation period.

The value of pH was 5.7 at the start. It decreased after 20 hours, reached a minimum, then increased to exceed the initial value.

The decolourization time of the resazurin solution showed a marked and well measureable change in the phase of exponential growth. It proved to be suitable for rapid indication of the exponential growth phase and, consequently, for the determination of the cultivation time of pre-cultures.

On the basis of kinetic investigations five phases of growth were observed. The maximum of the specific growth rate was 0.23 h^{-1} with a generation time of 3.1 h. The milk clotting enzyme formation seemed to be associated with growth and decreased with non-growth. Consequently, the production rate constant k_{p1} had a positive value and k_{p2} had a negative value. The latter seems to be closely related to non-growth as well as to the increase in pH.

The results obtained gave useful information both for further laboratory work and for industrial batch fermentation.

ACTION OF CHYMOSIN AND OF A MICROBIAL MILK CLOTTING ENZYME PREPARATION ON β-CASEIN

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Comparative experiments were carried out with crystalline chymosin and a purified rennet substitute derived from *Endothia parasitica* as developed in the authors' laboratory (Vámos-Vigyázó *et al.*, 1978b) to study the nature

of the differences in the actions of the two enzymes. Results obtained with whole, $\varkappa$- and α_s-caseins as substrates have been reported earlier (VÁMOS-VIGYÁZÓ *et al.*, 1980a, b, c). The present study gives an account on the enzymatic degradation of β-caseins prepared from raw (I), pasteurized (65 °C, 30 min, II) and hydrogen peroxide-treated (III) milks (VÁMOS-VIGYÁZÓ *et al.*, 1978a) as followed by polyacrylamide gel electrophoresis and non-protein nitrogen (NPN) determinations.

According to the electrophoretograms, pretreatment of milk decreased the resistance of β-casein to the so-called non-specific proteolytic action of chymosin. With the microbial enzyme, protein degradation was much more pronounced than with chymosin, irrespective of whether the substrate had been prepared from raw or pretreated milk. The initial inhibition of the microbial enzyme as observed with whole, α_s- and $\varkappa$-caseins prepared from hydrogen peroxide treated milk did not appear with β-casein. The number and relative electrophoretic mobilities of the breakdown products formed by the microbial enzyme from β-caseins I and II were identical and differed from those obtained from β-casein III.

During the first 60 min of the reaction practically no NPN was formed by chymosin from β-caseins I and II. With β-casein III the values increased up to 15 min, then the reaction came to a standstill. With the microbial enzyme, NPN formation from all 3 β-caseins continued up to the end of the observation period (60 min) and reached by that time a value 2–2.7 times that obtained for chymosin. The increase in NPN was highest with β-casein III and lowest with β-casein II. This indicates a slight inhibition of the non-specific proteolytic action of the microbial enzyme on β-casein from heat treated milk. These findings are in agreement with those observed on whole and $\varkappa$-caseins. α_s-Casein did not yield measurable amounts of NPN under similar conditions.

The results reported here confirmed the higher proteolytic activity of the microbial preparation as observed in cheese making (KISS *et al.*, 1975) and in the model experiments cited, revealing, at the same time, differences in the mode of action of the two enzymes on β-casein: the higher resistance of this protein to rennet action (TAM & WHITAKER, 1972) as compared to α_s-casein did not appear with the given microbial preparation.

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ENZYMATIC REACTIONS INFLUENCING THE LIPIDS OF FOODS

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Disruption of plant or animal tissues causes an enzymic breakdown of endogenous lipids. The initial event is a hydrolytic attack, releasing free fatty acids which are subsequently attacked by oxidative enzymes. Lipid-bound fatty acids are released by acyl hydrolase action as an initial step. Free fatty acids rarely occur in significant amounts in healthy plant and animal tissues, their concentration is low in the raw materials of food processing. The disruption of intact tissue leads to a breakdown of acyl lipids, predominantly of triacylglycerols and phospholipids with a resultant loss of their fatty acid constituents.

Conditions causing losses of acyl lipids:

– seed germination: hydrolysis of reserve triglycerides, loss of cellular membrane lipids,

– leaf senescence: loss of chloroplast lipids, increase of free fatty acids,

– fruit ripening: loss of galactolipids,

– cell disruption: physical damage, hence microbial infection.

Plant tissues contain active enzymes which may attack, during the destruction of the cells, the lipids of the membranes. The two main enzymes: lipolytic acyl hydrolase (LAH) and lipoxygenase (LOX) are involved in the breakdown of lipids in plant tissue but they are present in different subcellular fractions. Lipolytic acyl hydrolase (LAH) is not present in the plastids, mitochondria or microbodies, it occurs, however, in a low density fraction, which is close to the microsomal fraction (WARDALE & GALLIARD, 1977). Microbodies may readily lose some of their enzymes to the surrounding medium (LIPS, 1975). Lipoxygenase (LOX) which converts the unsaturated fatty

acids, linoleic and linolenic acid into their hydroperoxide derivatives, is very differently localised in plant tissues. LOX activity was measured in a wide range of subcellular fractions (lysosomal fractions, heavy body fractions with a density similar to plastids, chloroplasts and small vacuoles).

In some fruits and vegetables the processes occurring in disrupted tissues are of major importance as lipid degradation products cause a characteristic flavour. Volatile carbonyl compounds of C_6 and C_9 are formed by fatty acid breakdown in the fruits of tomato, cucumber, banana or the leaves of tea. The C_6 aldehydes are formed from C_{18} polyunsaturated fatty acids in a sequential enzyme system involving lipoxygenase which preferentially oxygenates at the 9-position, followed by a hydroperoxide cleavage system which is, however, specific for the 13-hydroperoxy isomers (GALLIARD & MATTHEW, 1977).

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THE ROLE OF INVERTASE IN MIXED CULTURES OF YEASTS

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The enzyme invertase (β-D-fructofuranoside fructohydrolase EC.3.2.1.26.) plays an important role in assimilating saccharose in yeast cells. In *Saccharomyces cerevisiae* the enzyme is bound to the outer surface membrane. Baker's yeast has a high invertase activity, on the other hand *Candida* species show a large variety of activities.

Laboratory experiments have been carried out applying the strain *Saccharomyces cerevisiae* VDH (BAKER'S YEAST FACTORY, Budafok) and wild yeast strains. The wild yeasts were isolated from samples taken from industrial fermenters used for baker's yeast production. Investigations have been carried out to study the morphological and biochemical character of the isolated wild yeast strains. Mono- and disaccharides were differently assimilated by the strain *Saccharomyces cerevisiae* VDH and the four isolated wild yeasts (Table 1).

The wild yeasts C–I–IV most frequently occurring in baker's yeast were proven to be *Candida krusei* strains showing no assimilation of saccharose. Efforts have been made in order to discover the occurrence of and increasing

Table 1

Auxanographic determination of sugar assimilation of the strains VDH and Candida I–IV

C- and N-source	VDH	C–I	C–II	C–III	C–IV
Glucose	+	+	+	+	+
Galactose	+	–	–	–	–
Saccharose	+	–	–	–	–
Maltose	+	–	–	–	–
Lactose	–	–	–	–	–
Raffinose	+	–	–	–	–
Potassium nitrate	–	–	–	–	–
Ammonium sulphate	+	+	+	+	+

infection by *Candida krusei* in baker's yeast when using molasses (high saccharose content) as main carbon source for yeast production.

Shaken flasks and laboratory glass fermenters were used for studying *Saccharomyces cerevisiae* VDH proliferation together with different concentrations of *Candida krusei* in mixed cultures (Table 2).

Table 2

Growth and biomass production of VDH and C–I wild Candida strain in pure and mixed cultures

Inocula	Sugar source	Generation time T_G (hours)	Biomass production (g l^{-1})
VDH	molasses	2.3	17.1
C–I	molasses	2.3	5.1
C–I	glucose	1.2	16.9
5% C–I/VDH	molasses	1.8*	24.1
15% C–I/VDH	molasses	1.4*	23.1

* Average generation time for mixed inocula of C–I and VDH

A very limited growth of *Candida krusei* was observed when grown on molasses, however it showed a dynamic growth when growing in mixed cultures together with the *Saccharomyces cerevisiae* VDH strain. In the course of the process C–I infection showed an increase from 5% to 57% and from 15% to 86%. It was concluded that the common infection of *Candida krusei*

in baker's yeast was due to its assimilating the glucose extracellularly inverted by *Saccharomyces cerevisiae*. On the basis of our experiments and measurements these phenomena were found to be the result of a symbiotic growth of the two yeast strains.

ENZYMES INVESTIGATED IN BIOCHEMICAL—PHARMACOLOGICAL WORK

I. ISOLATION AND PURIFICATION

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In the course of our biochemical—pharmacological work the following enzymes have been investigated: dopamine-beta-hydroxylase (DBH), phosphodiesterase (PDE) and cyclic nucleotide-dependent protein kinase.

DBH: the enzyme was isolated from bovine adrenal medulla. After homogenizing the tissue the nucleus fraction was sedimented and then the mitochondrial fraction was obtained by differential centrifugation. Solubilization of DBH from the chromaffin granules sedimenting together with the mitochondrial fraction involves hypo-osmotic treatment, thawing—freezing and detergent treatment. Subsequently the membrane fragments have to be removed. The enzyme was purified by ammonium sulphate fractionation, ionexchange chromatography and gel chromatography (Földes *et al.*, 1972).

PDE: The enzyme was isolated from rat heart and brain as well as from calf brain. The 70 000 × g supernatant of the tissue homogenate was salted out with ammonium sulphate saturated to 40%. The precipitate was dissolved in a slight amount of buffer and desalted by dialysis. Then DEAE-cellulose column chromatography followed with gradient elution. The fractions thus obtained were further purified by *Sepharose* 4B chromatography (Beavo *et al.*, 1970; Fujimoto *et al.*, 1974).

Protein kinase: binding proteins were isolated for the determination of cyclic nucleotides (cAMP and cGMP). The cAMP-dependent protein kinase was obtained from rabbit skeletal muscle or from bovine adrenal cortex, the cGMP-dependent protein kinase from locust. In both cases the preparation of a crude extract was sufficient, no further purification was necessary.

The isolation from rabbit skeletal muscle was performed in two ways:

– homogenization in EDTA solution, thereafter acid precipitation, ammonium sulphate fractionation and dialysis were applied (Miyamoto *et al.*, 1969).

– After polyethylene glycol fractionation the purification was carried out by DEAE cellulose chromatography, with gradient elution (Doskeland & Haga, 1978).

From the bovine adrenal cortex (Tsang *et al.*, 1972) and from locust (Wood *et al.*, 1976) the binding protein of suitable activity was obtained by a simpler method, *i.e.* fractionated centrifugation.

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ENZYMES INVESTIGATED IN BIOCHEMICAL–PHARMACOLOGICAL WORK

II. ACTIVITY MEASUREMENTS

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In the course of the isolation of the two enzymes described in the first part, *i.e.* dopamine-beta-hydroxylase and phospho-diesterase as well as in the isolation of the cAMP-binding protein and in the different phases of purification the following activity measurement methods have been applied to check the effect.

Measurement of the activity of dopamine-beta-hydroxylase (DBH)

– The underlying principle of the determination with tyramine substrate is the hydroxylation of tyramine to nor-synephrine by the enzyme. After the reaction had been stopped, the para-benzaldehyde formed upon the

oxidation performed in alkaline medium gives a sharp absorption at 330 nm and can be easily measured by spectrofluorimetry (CREVELING *et al.*, 1962; PISANO *et al.*, 1960).

– When applying dopamine substrate, the reaction is as follows:

$$\text{dopamine} + \text{ascorbic acid} + O_2 \rightarrow \text{noradrenaline} + \text{dehydroascorbic acid} + H_2O.$$

The noradrenaline formed was measured spectrofluorimetrically, after oxidation with potassium ferricyanide (LEVINE *et al.*, 1960).

Measurement of phosphodiesterase activity

The method of PÖCH (1971) was applied. The rate of hydrolysis of the labelled cAMP was determined after precipitation of the product (5-AMP) with $ZnSO_4$–$Ba(OH)_2$.Unit activity was defined as the quantity of cAMP expressed in nmoles which was hydrolyzed in 20 min to 5-AMP.

Determination of the cAMP-binding protein (protein kinase)

The measurement is made possible by the competitive binding between ^{3}H-cAMP and the binding protein which takes place in the presence of cAMP. After incubation at 2 °C for 90 min the free and protein-bound radioactivities can be separated.

– By precipitation with $(NH_4)_2SO_4$ (GEISLER *et al.*, 1977). From the water suspension of the precipitate obtained after centrifugation, radioactivity can be measured with *Instagel* (scintillation aid).

– By *Charcoal* adsorption (MADSEN *et al.*, 1975) after binding of the free ^{3}H-cAMP the radioactivity bound to protein can be determined from the supernatant.

With the aid of a calibration curve the unknown cAMP content can be determined.

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DETERMINATION OF THE PYRUVATE AND LACTATE CONTENT OF MILK WITH THE AUTOMATIC ANALYZER "CONTIFLO"

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In judging the quality of milk the hygienic characteristics are of primary importance. The microbial impurities of milk decisively affect the sensory properties as well.

According to the investigations of Tolle (1973) pyruvate occupies a central place in the metabolism of all the microbes but one that occur in milk and the amount of this metabolite is in close correlation with the microbial contamination of the milk. As pyruvate does not change upon the action of heat, its amount permits of conclusions as to the microbe activity prior to heat treatment.

In judging the shelf life of milk, beside the actual pyruvate content, also the changes occurring upon a short thermostating are conclusive.

Lactic acid producing bacteria are frequent contaminants of milk and their quantity can be measured on the basis of the lactic acid produced or its changes.

In the course of our investigations the procedure based on the principle of fluorimetry as developed by Heeschen (1969) has been adapted to the automatic analyzer *Contiflo*. An *Evans* fluorimeter equipped with a flow cuvette was applied as detector. The pyruvate content of commercial milks of different origin was determined in the range of 0–20 mg l^{-1} with an accuracy of ± 0.2, their lactate content in the range of 0–200 mg l^{-1} with an accuracy of ± 3 mg l^{-1}.

The behaviour of some milks during storage at different temperatures has been investigated, too.

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THE EFFECT OF HEAT AND COLD SHOCK ON THE MOLECULAR MASS OF THE PLASMA PROTEINS CARRYING THE MAXIMUM TTC-REDUCING CAPACITY OF YEASTS

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The distribution according to molecular mass of the water soluble plasma proteins and the molecular mass of the proteins carrying the maximum TTC reducing capacity of the mesophilic yeast strain *Torulopsis utilis* (T82) and the psychrophilic yeast strain *Candida utilis* (C9) grown in glucose media at their respective optimum growth temperatures (310 and 298 K) were compared upon the effect of heat (373 K, 30 min) and cold shock (83 K, 1, 3, 5 ×30 min).

The cell suspensions were disrupted by sonication and the water soluble plasma protein fractionated on the molecular sieve *Sephadex* G 200 in pH 7.2 TRIS buffer. The protein content of the fractions was determined by direct absorption spectrophotometry according to *Layne*. TTC-reducing capacity was determined in 0.05% TTC solution.

The changes occurring upon the action of the heat and the cold shock in the aggregation state of the water soluble plasma protein and the molecular mass of the protein carrying maximum TTC-reducing capacity were investigated as well.

Our experimental results can be summarized as follows. The TTC-reducing capacity of the untreated cells has maxima in the ranges of both 10^5 and 10^4 D (Daltons). Upon the action of heat treatment the amount of protein in the 10^5 D range decreased both in the psychrophilic and in the mesophilic yeast strain, while the amount of protein in the 10^4 D range increased. The molecular mass of the mesophilic strain carrying maximum TTC-reducing capacity in the range of 10^4 D showed a significant increase (association) during freezing of both the cell suspension and the water soluble plasma protein. The association is more expressed on freezing the plasma protein and is of the same extent after treatments repeated 1, 3 and 5 times, resp.

In spite of the considerable changes in protein profile on heat treating the plasma protein no significant change can be detected in the molecular mass of the protein molecule carrying maximum TTC-reducing capacity.

In the water soluble protein fraction of the psychrophilic strain the cold shock repeated 3 times, while in the case of the cell suspension only the cold shock repeated 5 times resulted in association of the molecular mass of the protein of maximum TTC-reducing capacity.

The comparison of the results of the freezing experiments shows that the state of aggregation of the water soluble plasma protein fraction is altered to a lesser extent by *in vivo* than by *in vitro* freezing.

An opposite trend can be observed upon heat treatment. Our results show also that the substance of the water soluble plasma protein of the psychrophilic strain is more resistant to both freezing and heat treatment than that of the mesophilic strain.

INVESTIGATIONS INTO THE RELATIONSHIP BETWEEN THE TTC-REDUCING CAPACITY AND THE PLASMA REFRACTIVE INDEX OF YEAST CELLS

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It is a known fact that in the case of cell decay the cell loses water and the concentration of the plasma increases. The concentration of the cell can be characterized by the refractive index of the cell as the specific refractive indices of the plasma components are very similar.

The objective of our work was to collect further data for following the decay of the cell. It arose that enzyme investigations might be suitable to characterize cells being in different stages and further informations could be gained if the results of the enzyme tests could be correlated to the refractive index of the cell.

Our investigations were carried out with cells of *Torulopsis utilis* T-82. In order to investigate the physiological state of the cells the determination of the reducing capacity of the cells was chosen. For quantitative estimation of the reducing capacity of living organisms oxidizing—reducing indicator dyes are suitable. TTC reduction forms a red formazan from triphenyltetrazolium chloride. The underlying principle of measurement is the formation of water insoluble red formazan from TTC by enzymes. The amount of formazan formed during a given time is the measure of the reducing capacity.

In order to make the method suitable for measurement it is necessary to activate the glucose dehydrogenase enzyme of the living cell with its substrate and to determine the pH value at which reducing capacity is optimal. The reducing capacity was measured at two different pH values; at pH 6.4. the optimum growth pH of the cells and at pH 7.4, favourable for the dehydrogenases. The experiments showed that of the two pH values investigated reducing capacity is stronger at pH 6.4 and the initial glucose concentration of 22.5 mg cm^{-3} is optimal.

From ulterior investigations it could be established that in the initial stage of the reduction process first a given amount of formazan is bound to the cells and the linear section of the reduction can be observed only after 0.5 h reaction time. If the reducing capacity is characterized by the amount of formazan in μmoles formed between the 1st and 2nd h, the TTC available is still sufficient and the interfering effect of the cell-bound formazan can be eliminated, too.

Thereafter we investigated the relationship between the reducing capacity of the cells and the plasma refractive index. The relationship between the two parameters is quadratic. In the range of refractive indices between 1.3824 and 1.3892 the living cells of high reducing capacity can be found, in the case of higher refractive indices as found with damaged and dead cells, the reducing capacity decreases and ceases, respectively.

ENZYMATIC PROPERTIES OF INDUSTRIAL INTEREST OF MYCOBACTERIA

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Three topics of our theoretical research work were selected which might be of interest as related to the practical utilization of *mycobacteria*.

1. The production of *mycobacteria* on hydrocarbons as carbon source.
2. N_2-binding capacity of *mycobacteria*.
3. Steroid transforming capacity of *mycobacteria*.

1. From the works of Foster (1962), Fredricks (1967) and Trust and Mills (1967) it became known that different kinds of *mycobacteria* are able to grow on media containing alcanes of 10–18 carbon atoms as carbon source. The metabolism of these occurs by terminal oxidation *via* alcohol, aldehyde and carboxylic acid which is then decomposed by beta-oxidation. In the course of these processes the enzyme isocitrate lyase is induced. The *mycobacteria* grow very well on alcanes of higher carbon atom numbers as well as on media containing mixed hydrocarbons (*e.g.* kerosene, especially *Diesel* oil, brake oil), while on hydrocarbons of low carbon atom number they do not grow at all or only slightly.

This property of the *mycobacteria* might be of interest in purifying petroleum products, waste water or in producing proteins from hydrocarbons.

2. It is known that numerous *mycobacteria* are able to fix nitrogen directly (*M. flavum*, *M. roseo album* 358, *M. sp.* 571). A unique property of these bacteria consists in the fact that they are able to fix nitrogen also in

acid soils. Their action requires also the presence of metal ions (Mo^{2+}, Co^{2+}, Mg^{2+}).

3. The capacity of some microorganisms to reduce 4-ene-3-oxosteroids has been known for long. The 5-α reductase responsible for the reduction has been detected in *M. smegmatis* by HÖRHOLD (1967).

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THE ROLE OF THE ALKALINE PHOSPHATASE TEST IN INVESTIGATING MILK AND MILK PRODUCTS

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In the alkaline phosphatase test of cacao-, chocolate- and caramel-containing milk as well as milk-containing ice cream or fruit-yoghurt the interfering effect of the basic colour of the products might be eliminated by the dialysis procedure.

On the basis of the similarity theory the conditions of the test have to be selected so as to ensure that at least 90% of the *o*-cresolphthalein set free in the test pass from the coloured medium into the dialysate. An essential feature of the dialyzing jacket is that in a medium of pH 9.6–10 it should have pores exceeding $10.74 \cdot 10^{-8}$ cm and at the same time smaller than the particles of the colouring matter of the product.

The alkaline phosphatase enzyme of milk is very heat-sensitive. Heat inactivation may occur at 65 °C in 5 min or at 70 °C instantaneously, depending on the physicochemical properties of the milk and the production technology applied, thus it is doubtful that the test indicates the decay of the pathogen in the case of cattle tuberculosis. These considerations make it necessary to apply a severe test in the investigations of milk destined for babies or school children.

The enzyme was isolated, purified and concentrated according to *Par. G. Lefranc* and *K. Han*, from the PASTEUR INSTITUTE, Lille.

The isolated enzyme was made up with physiological sodium chloride solution to the original volume of the sample and tested in parallel with the original sample (*i.e.* milk) under identical conditions. The results of colour measurements proved that the reaction can be evaluated better if carried out with the isolated enzyme than with milk.

As published also earlier, it was established in colour measurement in a tristimuli objective device that from the existing and planned as well as modelled 16 reactions the one carried out with *o*-cresolphthalein phosphate gives – under optimized and identical conditions – the reaction best evaluable. Similarly it has been mentioned that when applying the NH_4Cl–NH_4OH buffer in the concentration described, the phenol reaction with 2,6-dibromoquinone chloroimide as prescribed by the *International Dairy Standard (FIL–IDF 63, 1971)* cannot be estimated.

The substrate planned and existing in the form of a model was synthetized on the basis of the reaction according to *Gattermann* under the conditions prescribed.

Thereafter the reactions with 4-amino-antipyrine and *Folin–Ciocalteu* for phenol were revised in opaque and transparent media by colour measurement. It was established that under the conditions of the standard cited the *o*-cresolphthalein–lye reagent gives the best objectively evaluable colour reaction, moreover, *o*-cresolphthalein equivalent to 4 $\mu g\ cm^{-3}$ of phenol (taking into account the factor 2.4, this is equivalent to 17.66 μg and 35.32 $\mu g\ cm^{-3}$, resp.) can be detected in the milk itself, whereby the necessary equipment could be reduced and the test made applicable for use in dairy plants.

Thus the qualitative test could be quantified.

VARIANTS, OPTIMIZATION AND FIELD OF APPLICATION OF THE ACID PHOSPHATASE TEST OF MILK

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The acid phosphatase enzyme might be more suitable to control feed milks, feed milk powder or consumers' milk upon the order of the medical doctor or veterinary or to control heat treatment of sterile milk than the less heat stable alkaline phosphatase enzyme.

Therefore the authors have thoroughly studied the possibilities of application and optimization of the test.

In order to design the most suitable substrate and to model it milk of 0.001 IU cm^{-3}, succinic acid–sodium tetraborate buffer, thymol blue, cresol red and phenol were applied and during the colour measurements it was established that the order of the estimability of the reactions is as follows: thymol-blue acid, cresol-red acid, phenol-4-amino-antipyrine, phenol-2,6 dibromoquinone chloroimide.

In the course of the investigations it was established that the test as carried out with hydrogen—thymol blue phosphate was most suitable to detect the acid phosphatase enzyme of milk.

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PROTECTION OF ORANGES BY GAMMA RADIATION AGAINST *CERATITIS CAPITATA* WIED

I. FÉSÜS, L. KÁDAS and B. KÁLMÁN

(Received: 3 January 1979; revision received: 18 July 1980; accepted: 16 October 1980)

The eggs and larvae of *Ceratitis capitata* Wied. may be destroyed on oranges by treatment with gamma radiation. Out of doses of 0.2, 0.4, 0.6 and 0.8 kGy, 0.2 kGy was found in an earlier experiment not to be sufficient to kill all stages of development, while 0.8 kGy damaged the peel of oranges and caused softening to a substantial degree. This treatment affected the flesh of the fruit, too, although to a lesser degree. Treatments did not damage the ascorbic acid content of the fruit. After treatment with doses above 0.2 kGy, the insect was not able to finish its life-cycle. Death time decreased with increasing radiation doses.

An important result of the study was that, by treating oranges in quarantine with doses of 0.4–0.6 kGy, the insect was killed without damage to the fruit.

The marketing of tropical fruits in Hungary is strictly regulated by plant quarantine control specifications. One of the most important specifications is that tropical fruits infected by *Ceratitis capitata* Wied. must not be commercialized, or those received during the winter must be held in cold storage for 21 days. Thus it became necessary to find a protecting procedure which excludes the possibility of infection on the market.

KOIDSUMI discovered already in 1930 that an appropriate dosage of X-rays may be successfully applied to kill insects in fruits in quarantine. This problem, however, came to be thoroughly studied during the recent 25 years. The steadily increasing importance of tropical and Mediterranean fruits on the world market prompted research into new methods of protection against insects. The frequency of infection by various kinds of *Treptida* fruit flies, as yet not acclimatized to the importing countries, necessitated the search for an efficient method of disinfestation (STEINER *et al.*, 1965). Extensive investigations by BALOCK and CHRISTENSON (1956) proved that treatment with X-rays or gamma radiation may be applied to kill insects detrimental to fruits. CORNWELL in 1966 provides concrete data on the sensitivity of the different stages of development of *Ceratitis capitata* to ionizing radiations (Table 1).

His investigations were aimed at establishing efficient doses and the applicability of the method. In the treatment of foods, however, it is important whether a method, no matter how efficient in disinfestation, is causing biochemical, organoleptic or other qualitative changes in the food. On this problem, information could be expected from experiments aimed at extending the

storage life of oranges. The doses applied in these experiments, in consequence of their aim, were several times higher than the doses needed for disinfestation.

According to the investigations of MONSELISE and KAHAN (1968), changes caused by irradiation are affected by the degree of ripeness of the

Table 1

Sensitivity of the various stages of development of Ceratitis capitata *Wied. to ionizing radiations*

(CORNWELL, 1966)

	1-day-old egg	Pupa of 1–5 days	Immature larvae 1–3 days
LD_{95} (R)	1 450	1 850–2 700	1 800–2 400

fruit. Changes are least significant in the fully ripe oranges. With increasing dosages, the peel of the fruit is mainly damaged showing browning or sponginess.

BELLI-DONINI and co-workers (1974) and RIOV (1975) found also a peel-damaging effect when using 0.2 kGy for treatment.

SATTAR and co-workers (1970) treated oranges directly after harvesting with 0.5, 1 and 1.5 kGy and stored them for 60 days at 4–6 °C. When scoring for colour, consistency, flavour, taste and appearence, oranges treated with 0.5 kGy were found unanimously the best. Difference of any significance was not found in the ascorbic acid content and other chemical properties between the control sample and the one treated with 0.5 kGy.

In these experiments, radiation doses sufficient to kill *Ceratitis capitata* in all its stages of development while affecting least the fruit quality were selected.

1. Materials and methods

The fruit used in the experiments was of Italian origin (*Citrus sinensis* cv. Eliptic of May). A non-selected sample, characteristic of the whole consignment, was treated.

Irradiation was carried out in the RH-30 type Soviet self-shielded radiation source at the CENTRAL FOOD RESEARCH INSTITUTE. The active material of the irradiator was ^{60}Co of an actual activity of 0.44–0.48 PBq and a dose intensity of 4.2–4.4 kGy h^{-1}. Dose measurements were carried out on a *Fricke–Hart* system chemical dosimeter (KÁLMÁN. 1971).

1.1. Essay into mortality

Oranges were infested with the *Ceratitis capitata* culture obtained from the INTERNATIONAL ATOMIC ENERGY AGENCY. Sixty five oranges and 50 imagos of Orange flies (male and female, 1:1) were placed in isolators each. Subsequent to copulation eggs were laid by the females continuously on the oranges. Cultures were kept for 14 days in climatic chambers at 25 ± 1 °C. Imagos were removed from the cultures immediately prior to treatment. Thus, the oranges were infested with eggs and larvae at various stages of development. A few fruits were opened directly before treatment in order to establish the degree of infestation and the possible natural mortality of larvae. The number of eggs on one orange amounted to 8–36 and that of the larvae in the fruit to 11–27. The intact fruits of unknown inner infestation were treated with 0.4, 0.6 and 0.8 kGy, resp., in four repetitions each. One sample consisted of 5 fruits. Subsequent to irradiation mortality in eggs was established by microscopic investigations and the mortality of larvae by opening the fruits in day by day tests. Evaluation of mortality and the observation of control samples in the climatic chambers of 25 ± 1 °C continued throughout 1 month. Thus, the earliest death of eggs, young and grown larvae within the first hour after irradiation was established, in 3 repetitions, and the time in days required for the total destruction of the insect in all stages of development, in four repetitions. For the latter the error of the mean value was also calculated.

1.2. Chemical and physical analyses

In the course of these analyses, changes in the ascorbic acid content and in the texture of the fruit peel and flesh were followed up. Both tests were carried out on non-infested fruit within 24–48 h subsequent to radiation treatment. Fruit not irradiated was used as the control.

Ascorbic acid was determined in the juice pressed from the fruit flesh by titration with 2.6-dichlorophenol indophenol. For this test, the juice of 6 fruits each was homogenised and the average of four titrations was considered the result, given in mg per 100 g fruit flesh.

The texture of the skin and flesh was measured on a penetrometer Type OB–204 (manufactured by LABOR MŰSZERIPARI MŰVEK, Hungary). The internationally accepted 5-s plunging period was applied in the measurements. The texture of 5 oranges per each treatment was measured in 5 places on each fruit. Measurements on the peel were carried out along the perimeter belonging to the longest diameter, while on the flesh in the two halves of the fruit cut along the above perimeter. While measuring the texture of the fruit flesh, care was taken that the penetrating plunger should not touch the membrane between

segments but penetrate the flesh of loose texture. Thus the skin and flesh were measured with cones of different radius, corresponding to the consistency of the given part.

2. Results and conclusions

2.1. Mortality

In earlier unpublished studies 0.2 kGy radiation dose was found insufficient to kill all stages of development. Some of the well developed larvae entered into the pupal state and in one case even the fly developed from the pupa. That is why doses of 0.4, 0.6 and 0.8 kGy were applied. To evaluate results those stages of insect development were selected the radiation sensitivity of which was expected to be different. At these radiation doses, the final stage of development was never reached. Larvae were not hatched from the

Table 2

Observation of the first individuum of Ceratitis capitata *Wied., killed after treatment*

Radiation dose KGy	Serial number of tested repetitions	Beginning of mortality (h) in		
		eggs	immature larvae	mature larvae
0.4	1	48	120	360
	2	120	120	192
	3	168	168	288
	Mean value	112	136	280
0.6	1	48	120	264
	2	120	120	168
	3	120	120	216
	Mean value	96	120	216
0.8	1	24	48	192
	2	48	48	168
	3	120	120	168
	Mean value	64	72	176

Control: All eggs, larvae are alive and their entering in the pupa stage is continuous.

eggs and none of the larvae entered the pupa stage. The rate of mortality, however, differed with the dose applied.

The beginning of death in the various stages of development subsequent to irradiation is shown in Table 2, while the time required for the destruction of all stages is given in Table 3.

Table 3

Time required to the total death of Ceratitis capitata *in all its stages of development subsequent to irradiation*

Radiation dose (kGy)	Time required to total death (days) in		
	eggs	immature larvae	mature larvae
0.4	5.0±0.12	9.0 ±1.05	13.3±1.41
0.6	4.0±0.07	6.0 ±0.54	8.6±0.68
0.8	2.0±0.25	4.25±0.35	7.0±0.96

Control: In untreated fruits the insect developed normally. During the observation period (30 days) a total of 251 eggs, larvae, pupae were counted and the imagos swarmed continuously.

The results show two characteristic correlations, namely that, with increasing radiation doses, the process of mortality begins earlier and ends more rapidly and that, at the same dose level, there is no significant difference in the beginning of destruction in eggs and young larvae, while the destruction of grown larvae begins substantially later and takes more time. Because of its rapid killing effect 0.6 kGy seems to be the most expedient of the doses applied. At this dose level, the process of desctruction takes about 1 week and this is satisfactory, while the fruit is not harmed. In the control infested samples the development of the orange fly was continuous, their swarming undisturbed and natural mortality was not observed.

2.2. Chemical and physical tests

Changes in the ascorbic acid content subsequent to radiation treatment are shown in Table 4. Characteristic change in the ascorbic acid content consequent to different treatments was not found, differences are due to the heterogeneous character of the samples.

The consistency of the orange peel and flesh as a function of radiation dose is shown in Fig. 1. (Because different plungers were used for measuring the consistency of peel and flesh, the values obtained show only the trend of change and are not comparable.)

Table 4

Ascorbic acid content of oranges subsequent to treatment with various radiation doses

Radiation dose (kGy)	Ascorbic acid (mg per 100 g)
0 (control)	42.6±0.38
0.4	47.0±0.47
0.6	40.8±0.27
0.8	44.2±0.31

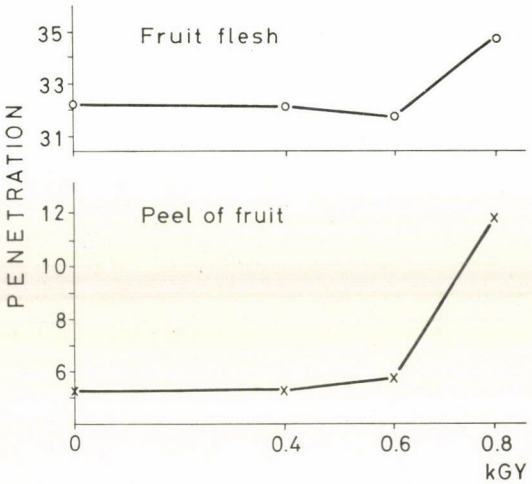

Fig. 1. Consistency of oranges as affected by gamma radiations

In the fruit treated with 0.4 or 0.6 kGy, no systematic or significant change was observed. Treatment with 0.8 kGy, however, caused substantial softening of the peel. The penetration value in the peel of these oranges is the double of that measured in the other samples. The change in the consistency of the fruit flesh is not so extensive. The present observations correspond to those found in the literature, since, however, behaviour during storage was not studied, browning and sponginess of the skin was not observed.

Oranges treated with different radiation doses were subjected to organoleptic testing, too. Panel members found no significant difference between the samples, but their number being low (six members) it would be desirable to organise more extensive tests.

Unanimous conclusion of this study is that doses of 0.4–0.6 kGy are suitable for satisfactory disinfestation of oranges in quarantine without affecting their quality.

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CHARACTERIZATION OF CERTAIN PROTEINS OF ANIMAL AND PLANT ORIGIN BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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(Received: 21 September 1979; revision received: 10 March 1980; accepted: 6 April 1980)

Utilizing the natural buffer system, a protein extract was obtained from muscles by pulping with water (1:3); plants were homogenized and the cytoplasmic juice was pressed out; the polyacrylamide gel electropherograms of the two materials thus obtained were evaluated. The analyses carried out under varied conditions prove the versatility of polyacrylamide gel electrophoresis (PAGE). The soluble protein fractions of muscles obtained from identical parts of animals of the same species and age proved very similar. Pork and beef may be distinguished on the basis of the myoglobin fractions of their proteins. It is of interest that the soluble protein fractions of the leg and breast of chicken differ significantly. The effect of heat treatment by different techniques, as a function of the extent of denaturation, could mostly be demonstrated by PAGE. The electropherograms of beef and egg-white and the 3:1 mixture of the two show that additioning of the bands does not occur upon mixing, however, they permit characterization of a certain sense.

The electropherograms of the various vegetables show a marked difference and thereby they are suitable for the characterization of the vegetables. Changes in the quantity of soluble proteins in vegetables during storage may easily be followed up with PAGE technique.

Gel electrophoresis – the two better known variants of which are polyacrylamide and starch gel electrophoresis (GORDON, 1969) – is particularly suitable to demonstrate the main relations in the intricate colloidal system of proteins.

Polyacrylamide gel electrophoresis, mainly used in analytical work in two different forms, provides to a certain extent different advantages. In one of them, gel columns in the other, gel plates are used for separation.

The desirable accuracy and reproducibility is ensured by disc electrophoresis as developed by ORNSTEIN (1964) and DAVIS (1964). This technique was used in the present work, too. The advantages of this technique are stressed by the summary reviews of ZWARTZ (1970) and KERESE (1975) as well as by many papers discussing problems related to food science.

Favourable experiences were reported by FARKAS and BENCZE-BŐCS (1969) and VASS-BALKAY and co-workers (1969) who used this technique in checking changes in the behaviour of protein fractions upon the application of a new method of preservation, the combination of heat treatment and irradiation. Similar experiences were reported by FARKAS-CSENTES (1970) in relation to the proteins of milk, egg and wheat. The paper of BECZNER-HEGYESI (1972)

reports on the characteristics of microbial and food proteins. The methodological research of GAÁL and coworkers (1973) highly promoted the use of PAGE in Hungary. The method was applied by TÓTH (1971; 1976; 1978) to the special field of enology. MIKLÓS-JUHÁSZ and TÖRLEY (1979) used it to detect the free amino acid and protein composition in grape varieties cultivated in Hungary; VÁLAS-GELLEI (1977) succeded in detecting milk and soy protein in the presence of meat protein by PAGE. BÉKÉS and VÁRADI (1976) used it to determine the purothionine content of Hungarian wheat flours. NEDELKOVITS and TRAN TRUYEN (1978) studied by PAGE the proteins in powdered milk prepared by different methods.

In the present study the muscle proteins of various animals were investigated in the raw state and after different heat treatments. A wide range of vegetables were studied for their characteristic protein fractions. Investigations related to milk are not discussed here because they were published in a number of papers, *e.g.:* PHAM VAN MINH and KÁDAS (1978) PHAM VAN MINH and co-workers (1978), PHAM VAN MINH and LINDNER (1978) and KÁDAS and co-workers (1979).

1. Materials and methods

1.1. Materials

Meat samples were taken from the back muscles of beef and pork (longissimus dorsi, sirloin, chop), leg and breast of chicken, all free of bone and skin.

The following vegetables were studied: carrots, parsley root, white cabbage, savoy and spinach as obtained on the market.

1.2. Preparation of the samples

A sample of a few g was cut out of various parts of the muscle selected. Twenty-five were then homogenized with 75 cm^3 distilled water. The supernatant was filtered through a qualitative filter paper and 25 mm^3 of the filtrate were used for PAGE.

The vegetables were cleaned in the customary way, then homogenized in a *Turmix* apparatus and pressed in a press lined with linnen. The cytoplasmic juice thus obtained was used for the investigations.

1.3. Electrophoretic test

All the experiments were carried out on the *Model* 69 apparatus, manufactured by REANAL, Budapest and the chemicals suggested and commercialized by the same firm, were used:

The electrophoresis was carried out in a gel of 7.5% related to the monomer. The buffer used was TRIS-glycine of pH 8.3 at a tube amperage of 2 mA, 5±1 °C applying a running period of 3.5-4 h.

Samples were applied directly with a micro-pipette to the surface of the gel used for running, omitting the sample gel. It was then blocked, avoiding mixing, with the buffer.

From the serum of animal tissues 20 mm^3, from all the vegetable juices 100 mm^3 were applied to the gel columns.

In order to visualize the protein fractions, the gels were dyed, subsequent to electrophoresis, with *Amido Black* for 15 min. The dye not adsorbed was removed by multiple washing with a 7% acetic acid solution.

1.4. Evaluation of the electropherograms

1.4.1. Visual evaluation. The visual evaluation of the gel column showing 7–9 dyed spots of the main protein fractions is possible if the amounts of protein applied to the columns and the conditions of the test are identical. A solution of this plan is given in the schematic diagram (Fig. 1) showing the photographs of the proteins belonging to various animal species.

Diagrams of the electropherograms of sarcoplasm samples taken from different animals are seen in the figure. They reflect the strength of the displacement parameters and of the protein fractions by the thickness of the lines, expressed in mm. As it may be seen in the schematic diagram the proteins separated by PAGE on the column may be represented, true even to the pertinent photographs *(a, c, e)* and are evaluable in their proportions (Fig. 2).

The electropherograms transformed to 100 mm (from the outside edge of the first protein fraction to the outside edge of the last protein fraction) proportionate distance and band width may be characterized by means of the

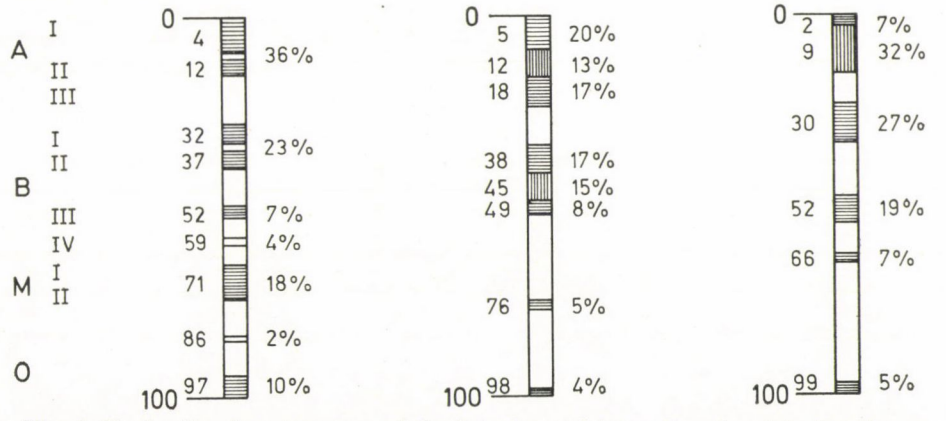

Fig. 1. Evaluation in percentage of the location and intensity of protein fractions

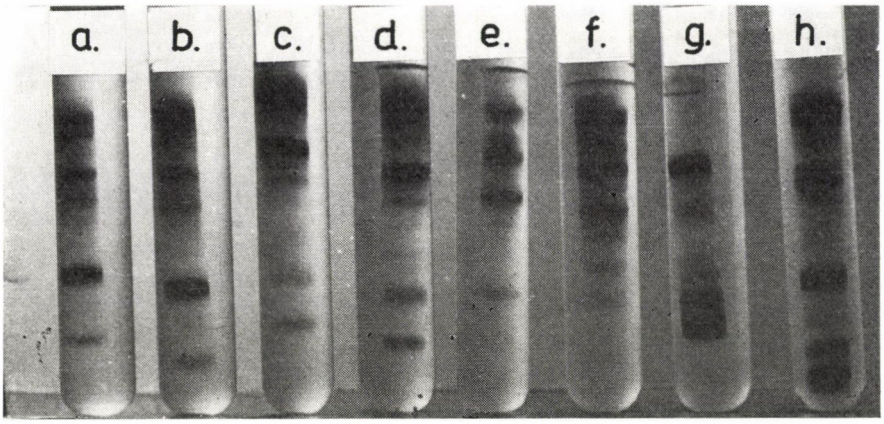

Fig. 2. Electropherograms of raw meat samples
a) Tenderloin of beef
b) Round of beef
c) Tenderloin of pork
d) Pork ham
e) Leg of chicken
f) Breast of chicken
g) White of egg
h) Beef and egg-white

percentual values as shown in Fig. 1, they may be even quantitatively evaluated on an approximative basis. The process is particularly valuable in the determination of trends in technological processes.

1.4.2. Instrumental evaluation. More accurate, suitable also for the numerical evaluation of quantitative correlations is the characterization by means of the area enclosed by the base line of the instrument and the densitograms of the gel columns, the absorption curves of the individual protein fractions. A densitogram taken by a *Chromoscan* type (manufactured by JOYCE & LOEBL, England) instrument from the gels shown in Fig. 2b is given

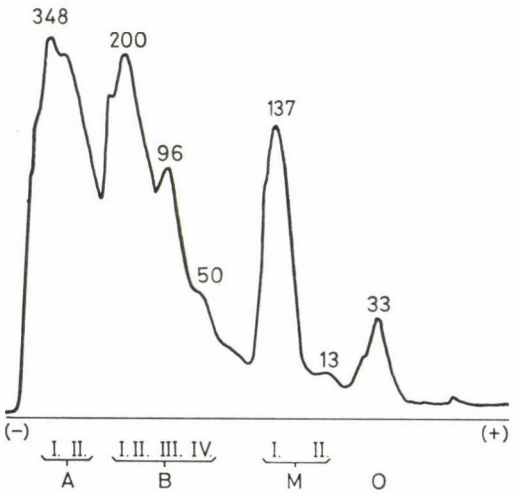

Fig. 3. Densitograms on the basis of beef sarcoplasm electropherograms

in Fig. 3. Although the results obtained by PAGE may be considered only semi-quantitative, the very close results, as seen in Table 2, prove the high reproducibility of the technique.

1.5. Determination of the soluble proteins

In order to be able to evaluate the relative proportions of protein fractions obtained by PAGE, the quantity of soluble proteins was also determined.

The meat sample was homogenized with distilled water (1:1) and 10 cm^3 of the filtrate were mixed with 10 cm^3 of 10% trichloro acetic acid, this was allowed to stand for 1 h, then centrifuged and washed 3 times with 5% trichloro acetic acid. The nitrogen content of the precipitate was determined by *Kjeldahl*'s method and the result multiplied by 6.25 to obtain the amount of soluble protein.

In the densitogram of beef sarcoplasm, taken by the *Chromoscan* densitometer 9 protein fractions could be distinguished. The fractions located closely were considered a group and marked with a letter enabling the comparison of values when for some reason, *e.g.* technological intervention, the individual fractions could not be perfectly separated.

The peaks and the area enclosed by them with the base line, *viz.* the light-absorption capacity of the individual protein fractions is dependent only partly on the amount of protein, it depends also from its dye-binding capacity (affected also by the rearrangement of the protein molecule upon heat treatment). Therefore, it seemed expedient to express the proportion of areas as a percentage of the total integrated numerical values found for all the peaks.

Since the protein fractions are identified on the basis of their distance from the start and their serial sequence (proportional to the molecular mass), only the mioglobin (*M*) and mialbumin (*O*) can be recognised with certainty because of their colour perceivable without dyeing. They could also be identified on the basis of data in the literature. The groups of protein fractions marked *A* and *B* consist probably of proteins of high molecular weight, of low motility and of the globulin type. In view of the aim of this study — differentiation (plant proteins) and following up to technological operations (meats) — this system of identification and the pertinent measured values proved to be satisfactory.

1.6. Meat preparation

Meats were prepared by the following methods:
- fried in teflon coated pan,
- baked in aluminium foil,
- baked by high frequency, to rare, medium rare and well done.

The fractions of the soluble protein separable by PAGE, their ratio, their losses upon denaturation, their sensory properties, their loss upon cooking and the amount of water-soluble protein content, were investigated. The method of protein extraction was not modified since FRATI and co-workers (1971) carried out PAGE tests in this way from cooked meats evidently not denaturated. Proteins completely denatured may be extracted by the technique developed by MACKIE (1968) by means of urea and this even permits the identification of the animal species.

In using a teflon-coated frying pan, only the surface of the pan should be greased lightly.

The aluminium foil used for baking was manufactured by KÖBAL (Kőbányai Könnyűfémmű, Hungary), for domestic use. The meat was wrapped directly in two layers of the foil. Cooking was done on a gas-range with directed flame.

The high frequency oven used was a *Philips* HN 1108 type product operated with a time automat.

In all three methods of cooking, meat cuts of 15 mm thickness were used. The required degree of doneness was established in preliminary cooking tests.

2. Results and discussion

2.1. PAGE tests of animal proteins

In the first year of the introduction of PAGE, it was considered suitable for the characterization of meats on the basis of their hemoglobin or mioglobin content (NAKAMICHI & RAYMOND, 1963; SMITH & EWATT, 1967; HOEYEM & THORSON, 1970). In Hungary VASS-BALKAY and co-workers (1969) and BECZNER-HEGYESI (1972) realized early the usefulness of the method.

The analysis of protein fractions in muscle sarcoplasm and the separation of soluble proteins by PAGE was started by PERRIE and PERRY (1970) and EBERMANN and BARNA (1972). FRATI and co-workers (1971) succeeded in detecting a substantial difference in the PAGE pattern of raw and heat-treated meats. Thus, it seemed obvious to proceed with these experiments.

In the course of developing processing technologies and in their checking in food manufacture and particularly in the catering industry, it is necessary to identify muscle tissue proteins quickly and reliably and keep interferences (*e.g.* heat treatment) at the same level. On the basis of denaturation, PAGE seems to be a suitable method for the above purpose. In relation to meat the following investigations were considered necessary:

- Characterization by PAGE of the meat of different animal species.
- Distinguishing between proteins from anatomically different parts of the same animal.

- Comparison of the electropherograms of muscle tissue types of different animals.
- Establishment of the denaturing effect of various technologies as used in the food and catering industries.
- Analysis by PAGE of muscle tissue and egg-white mixtures.

2.1.1. Characterization by PAGE of the meat of different animal species. It is considered the minimum interference to dilute sarcoplasmic proteins with distilled water subsequent to crushing the muscle cells and thus, dissolved in their own buffer system, to separate them by filtering from the solid, insoluble muscle tissue. Recently, increasing numbers of investigators applied this technique, *e.g.* LAAKKONEN and co-workers (1970), DESCHREIDER and MEAUX (1969). Buffers and solvents of digestive effect are used mostly with denatured proteins becoming insoluble on various grounds.

In the first step, protein from sirloin, pork chop and leg of chicken were fractionated by PAGE. The values obtained by measuring the area below the individual peaks related to the individual protein fractions are shown in Table 1.

Table 1

Numerical values belonging to the densitograms of raw muscles

		I.	*A*	II.	I.	II.	*B*	III.	IV.	I.	*M*	II.	*O*
Rump steak	I.V.[a]		348			200		96	50		137	13	33
	%[b]		39.8			22.9		11.0	5.7		15.3	1.5	3.8
Pork chop	I.V.	126		176		252			126		50		27
	%		39.9			33.3			16.6		6.6		3.6
Breast of chicken	I.V.	195		499		185		37			15		–
	%	21.0		53.7		19.9		4.0			1.6		–

[a] I. V. = Integrated value of the area measured under the individual peaks
[b] % = The area under individual peaks as percentage of the total area

The electropherograms of muscle tissue taken from anatomically identical parts of beef and pork and their densitograms are similar and thus may be considered parallel. The most characteristic difference lies in the quantity of the protein fraction marked *M*. In rumpsteak, about 16% of the total light absorption pertains to these fractions, while only 6.6% in pork chop. This corresponds to data found in the literature, according to which the mioglobin content of beef back muscle may be 8 times higher than that of pork. The breast of chicken contains – as revealed by its colour – less mioglobin than pork meat. In the present study, it was found to form 1.6% of the total light absorption.

In the investigated muscle of beef and pork, the proportions of the other protein fractions are nearly identical, thus fraction marked *O*, too, which is not even detectable in chicken breast. The proteins of chicken differ substantially from the former. Fraction *A* amounts to 70%, causing fraction *B* to shift in the direction of fraction *M*.

Thus already the preliminary tests proved the suitability of the methods used to distinguish the protein of different animal species. However, further investigations were necessary to establish the identity of electropherograms of the proteins coming from different individuals of the same species and to study the difference between tissues carrying out different biological functions.

2.1.2. PAGE of different individuals of the same animal species and of tissues from parts of the same animal. Samples of beef and pork were taken at one of the slaughterhouses in Budapest from the production line. The chickens were taken from the retail trade. They were broilers of about 1.3 kg, freshly slaughtered and electrophoresis was carried out on two parallel samples.

The beef was freshly slaughtered, precooled, *i.e.* ripened. The raw sample was taken every time from the left side of the fattened animal, using the commercial terminology from rumpsteak, sirloin and round steak.

Pork samples were also taken from the left side muscles of freshly slaughtered, well developed, precooled animals, anatomically from the same parts as with beef, according to the commercial terminology: from chops, tenderloin and ham.

Chicken samples were taken from the breast and leg as most characteristic muscles.

Values obtained on the basis of densitograms are given in Table 2.

The characteristic differences between animal species are seen in the table and in the photographs. The differences between individuals of the same species were not significant. The electropherograms made with the extracted sarcoplasm of animals *a* and *b* differed only within the limits of error inherent in the method, both for beef and pork.

The most uniform were the electropherograms obtained with beef muscles taken from various parts of the same animal, although some of the protein fractions (*B* IV, *M* II, *O*) provide the possibility of characterization by anatomical location. In the case of pork there is more chance of using the protein fractions for the characterization of anatomical location (*A* I, II, III; *B* I, II, III, IV; *M* I; *O*). This possibility was discovered by KERESE (1975). It is easy to distinguish between the breast and leg of chicken, because each of the protein fractions has a special character, particularly fractions *M* and *O*.

2.1.3. Characterization of heat treated muscle proteins. Both in frying in a teflon-coated pan and in baking in aluminium foil, the samples were turned

Table 2

Soluble protein fractions in raw meat samples

Meat sample		Protein fraction (%)									
		A			*B*				*M*		*O*
		I.	II.	III.	I.	II.	III.	IV.	I.	II.	
Beef	Sirloin		39.8		22.9		11.0	5.7	15.3	1.5	3.8
	Tenderloin of animal *a*		33.5		18.2		14.4	9.2	15.2	3.5	6.0
	animal *b*		37.0		16.4		8.7	9.9	17.2	3.8	6.5
	Round of beef animal *a*		36.8		18.0		13.1	7.0	16.1	3.5	5.5
	animal *b*		36.8		15.7		13.8	7.9	15.0	3.4	7.4
Pork	Chop	16.6	7.7	15.6	33.3		16.6	16.6	6.6	–	3.6
	Tenderloin of animal *a*	26.9	14.6	14.4	16.3		17.4	7.1	1.6	–	1.7
	animal *b*	26.9	14.8	14.3	14.1		17.4	8.1	2.8	–	1.6
	Ham of animal *a*	25.8	14.9	13.7	20.9		8.5	7.5	5.4	–	3.3
	animal *b*	25.0	16.1	15.3	20.2		13.3	10.3	3.2	–	1.6
Chicken	Breast of animal *a*	1.2	19.8	53.7	19.9	4.0	–	–	1.6	–	–
	animal *b*	1.1	25.2	46.8	22.0	6.2	–	–	1.2	–	–
	Leg of animal *a*	3.6	20.9	43.2	24.2	8.7	–	–	–	–	0.2
	animal *b*	4.4	13.6	41.5	30.5	9.4	–	–	–	–	0.3

over at half time in order to have the meat done on both sides. The samples were then cooled on a cold china plate. Under high frequency, the cuts were uniformly heated in their whole cross section, thus it was not necessary to turn them over. Treatment times and other technological parameters are given for beef in Table 3, for pork in Table 4 and for chicken in Table 5.

Table 3

Preparation and sensory properties of beef

Meat cut	Mode of preparation	Cooking time (s)	Sensory properties	Weight loss upon cooking (%)
Tenderloin	in aluminium foil medium rare	210	tender, pleasant taste (reminding of veal)	7.9
Tenderloin	in teflon-coated pan medium rare	210	slightly browned, inside pink, pleasant baked taste	8.2
Tenderloin	high-frequency medium rare	80	well done, however, of raw taste	8.8
Round of beef	in alum. foil medium rare	300	slightly leathery, reminding somewhat of veal	6.9
Round of beef	in teflon-coated pan medium rare	240	characteristic beef consistency and flavour	7.8
Round of beef	high-frequency medium rare	80	less done than the traditionally rare, blood red, of raw beef taste	7.3
Sirloin	high-frequency medium rare	80	well done, yet of rare taste	8.5
Sirloin	in aluminium foil medium rare	210	browned, leathery of raw blood taste	5.5
Tenderloin	in aluminium foil	180	surface gray, inside bloody, or row taste, fibrous consistency	5.8
Tenderloin	in teflon-coated pan rare	120	slightly leathery, bloody, of pleasant baked taste	7.0
Round of beef	in teflon-coated pan rare	120	slightly leathery, bloody, of pleasant taste	6.6

2.1.3.1. Beef prepared by different technologies. – Table 3 shows the technology of preparation and data of sensory evaluation. Table 6 contains the soluble protein fractions of treated beef (*A*, *B*, *M* and *O*), obtained by integrating the densitograms as seen in Fig. 4 as well as the degree of denaturation of the soluble proteins.

2.1.3.2. Pork prepared by different technologies. – The technology applied and data of sensory evaluation are given in Table 4. The soluble protein fractions, obtained by integrating the densitograms are shown in Table 7.

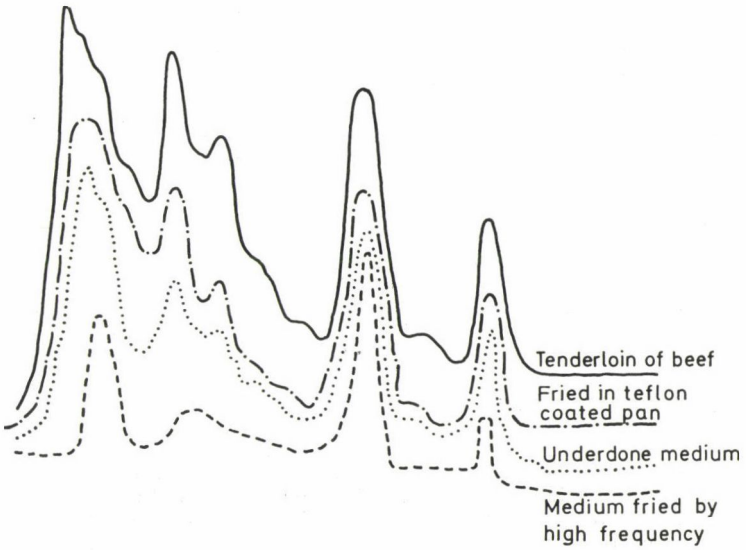

Fig. 4. Densitograms of beef tenderloin prepared in different ways

Table 4

Preparation and sensory properties of pork

Meat cut	Mode of preparation	Cooking time (s)	Sensory properties	Weight loss upon cooking (%)
Tenderloin	in aluminium foil medium rare	270	consistency characteristic of done meat, taste of raw meat	7.2
Tenderloin	in teflon-coated pan medium rare	90	slightly browned, inside pinkish, taste characteristically baked	6.5
Tenderloin	high-frequency medium rare	80	half done, very leathery, of elastic consistency and of slightly raw taste	10.7
Ham	in aluminium foil medium rare	270	half done, very leathery of elastic consistency and of slightly raw taste	6.5
Ham	in teflon-coated pan medium rare	120	light brown, inside pinkish, slightly leathery of baked taste	7.3
Ham	high-frequency medium rare	80	half done of leathery consistency, taste slightly raw	9.5
Chop	high-frequency medium rare	80	consistency well done, inside pinkish, taste slightly raw	9.4
Chop	in aluminium foil medium rare	210	appearance and taste reminding of underdone meat, consistency fibrous	5.0

Table 5

Preparation and sensory properties of poultry

Part of body	Mode of preparation	Cooking time (s)	Sensory properties	Weight loss upon cooking (%)
Breast	in aluminium foil medium rare	300	unevenly done, leathery, surface reminding of boiled meat, slightly raw	5.0
Breast	in teflon-coated pan medium rare	90	characteristically browned surface, taste well done	9.3
Breast	in high-frequency oven medium rare	40	appearance of boiled meat, taste characterless	10.5
Leg	in aluminium foil medium rare	300	unevenly done, surface reminding of boiled meat, leathery, taste slightly raw	5.6
Leg	in teflon-coated pan medium rare	120	surface scantly browned, inside fibrous, slightly leathery, taste baked	3.5
Leg	in high-frequency oven medium rare	40	consistency and taste of boiled meat, slightly stringy	8.5

Table 6

Soluble protein fractions in beef prepared in different ways

Meat cut	Mode of preparation		Protein fraction (%)				Denatured soluble protein (%)
			A	*B*	*C*	*D*	
Tenderloin	raw		33.5	41.8	18.7	6.0	0
	rare	in teflon-coated pan	39.1	34.1	21.6	5.1	18
		in aluminium foil	39.1	33.8	22.0	6.3	15
	medium rare	in teflon-coated pan	39.3	28.2	25.5	7.0	22
		in aluminium foil	32.7	22.9	34.8	9.6	18
		in high-frequency oven	24.1	24.7	46.0	5.2	35
Round of beef	raw		36.8	38.1	19.6	5.5	0
	rare	in teflon-coated pan	38.9	34.5	20.6	5.1	15
	medium rare	in teflon-coated pan	34.8	30.5	27.7	7.0	39
		in aluminium foil	39.7	20.6	35.5	10.0	43
		in high-frequency oven	50.5	17.5	28.0	4.0	40
Sirloin	raw		39.8	47.6	16.8	3.8	0
	medium rare	in aluminium foil	52.8	17.2	24.4	5.6	23
		in high-frequency oven	55.7	8.0	36.3	0	36

2.1.3.3. Chicken prepared by different technologies. – Table 5 contains the data on technology and the results of sensory evaluation. The soluble protein fractions of the treated chickens, obtained by integrating the densitograms, are shown in Table 8.

Table 7

Soluble protein fractions of pork prepared in different ways

Meat cut	Mode of preparation	Protein fractions (%)				Degree of protein denaturation (%)
		A	*B*	*C*	*D*	
Tenderloin	raw	59.3	37.5	1.4	1.7	0
	medium rare in teflon-coated pan	63.8	36.2	0	0	42
	in aluminium foil	60.9	25.2	9.6	4.3	25
	in high-frequency oven	56.6	41.7	1.7	0	55
Ham	raw	41.0	50.2	5.5	3.3	0
	medium rare in teflon-coated pan	62.6	35.2	1.4	0.8	50
	in aluminium foil	52.8	34.2	8.4	4.6	50
	in high-frequency oven	64.0	36.0	ny	0	50
Chop	raw	39.9	49.9	6.6	3.6	0
	medium rare in aluminium foil	71.3	23.9	1.8	3.0	40
	in high-frequency oven	97.2	2.8	0	0	63

Table 8

Soluble protein fractions of chicken breast and leg prepared in different ways

Body parts	Mode of preparation	Protein fractions (%)				Degree of protein denaturation (%)
		A	*B*	*C*	*D*	
Breast	raw	74.3	24.3	1.4	0	0
	medium rare in teflon-coated pan	85.3	14.7	0	0	35
	in aluminium foil	81.2	18.2	0	0	20
	in high-frequency oven	0	0	0	0	50
Leg	raw	65.7	33.3	0	1.0	0
	medium rare in teflon-coated pan	59.0	40.0	0	1.0	20
	in aluminium foil	52.1	47.9	0	0	22
	in high-frequency oven	0	0	0	0	56

As can be seen in the tables, the time of treatment highly affects denaturation and thereby the soluble protein fractions. For instance the densitograms of beef prepared by different technologies are all of the same character (Fig. 4), however, the areas under the peaks differ significantly as a function of treatment time. The only densitogram differing substantially is that of the meat treated in a high-frequency oven. The distribution of heat is uniform in the whole cut, therefore, it is not possible to bake it rare or medium rare. This is reflected in the densitogram. Baking under high frequency causes the highest degree of denaturation in the shortest time as compared with meat prepared by other technologies.

Table 9

Soluble protein fractions in the mixture of beef and white of egg prepared in different ways

Sample	Mode of preparation		Protein fractions (%)				Degree of protein denaturation (%)
			A	*B*	*C*	*D*	
Minced round of beef	raw		36.8	38.1	19.6	5.5	0
White of egg	raw		0	48.3	7.1	44.6	0
Mixture (minced meat: white of egg 3:1)	raw		21.5	14.3	28.6	34.7	0
Mixture	medium rare	in teflon-coated pan	0	0	55.9	44.1	49
		in aluminium foil	28.6	8.5	30.4	32.5	44
		in high-frequency oven	0	0	0	0	63
	well done	in aluminium foil	0	0	0	0	64
		in high-frequency oven	0	0	0	0	65

2.1.4. Electropherograms of meat mixed with egg-white. The aim of these experiments was to find out whether PAGE could be used to control cheaper proteins when used in meat products for enrichment.

Three parts of minced beef and one part of white of egg were mixed to form an elastic mass, which could be easily shaped into slices of $60 \times 100 \times 15$ mm. These slices were treated as shown in Table 9.

There is a substantial difference between the soluble proteins of meat and those of the white of egg. The densitogram of the mixture is quite different from what might be expected. Some of the peaks are shifted, and become deformed, others disappear completely (*A* and *B*). This is possible only if some structural binding of different strength occurs between the protein fractions. This phenomenon needs to be further explored.

2.2. Electrophoresis of vegetable proteins

2.2.1. Soluble protein fractions of various plants. In the first quarter of this century it has been proved that the proteins present in plant tissues are not homogeneous substances but may be separated into components (OSBORNE, 1924). With the development of analytical methods more and more protein fractions could be separated. At first only 4-10 components could be distinguished and these belonged probably to albumin and easily soluble globulin type simple proteins (KORPÁCZY, 1956). Today with the available refined techniques many more fractions, often 25–30 fractions can be separated.

In the analysis of the soluble proteins of plants PAGE plays an increasingly important part. It is used for the following up of the growth of individual

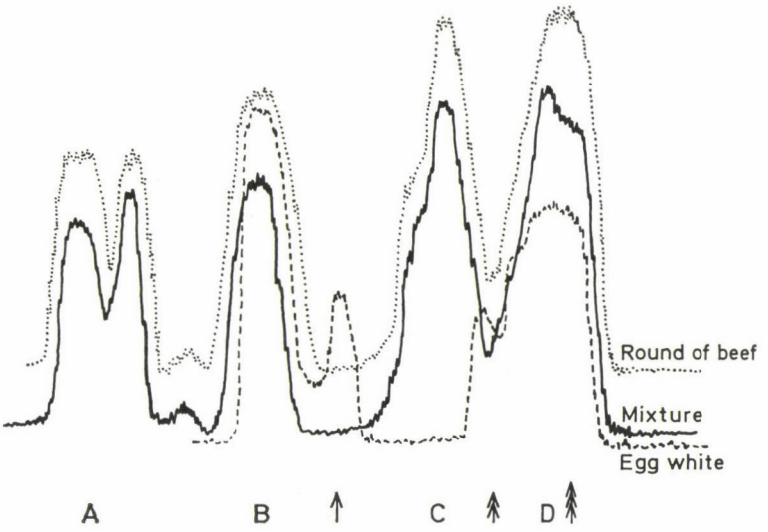

Fig. 5. Densitograms belonging to round of beef, egg-white and the 3:1 mixture of the two

plants, to prove genetic differences, to study enzymes and for many other purposes (STEWARD & BARBER, 1964; CLEMENTS, 1965; HALL *et al.*, 1972; BALÁZS *et al.*, 1973; BELEA *et al.*, 1975; MOLNÁR, 1973; 1975).

2.2.2. Characterization of different vegetables on the basis of their dissolved protein fractions. First the pressed juice of vegetables was studied to see whether they could be distinguished on the basis of the electropherograms of their protein fractions. Many kinds of vegetables were investigated. The experiences gained are summed up in Fig. 6 and Fig. 7.

In Fig. 6 the electropherograms of carrots and parsley roots are shown. Their densitograms are contained in Fig. 7. A glance at the Figures shows that the electropherograms of these vegetables differ substantially. An even greater difference may be seen in their densitograms. The amount of soluble proteins is substantially higher in parsley roots and there is also a characteristic difference in the quality of the protein fractions. The electropherograms of carrots shows 6, that of parsley roots 10 well distinguishable protein fractions. The proportion of the fractions is different, too. The most significant is the difference in the pair of an intense and a subfraction (marked in the densitogram: IX–X) in parsley root and the much smaller corresponding fraction in carrots.

The possibility of using this rapid method for distinguishing vegetables is proven by the densitograms of white cabbage and savoy which are subspecies of the same species (Fig. 8).

The electropherograms of the two kinds of cabbage are similar, in both of them 9 fractions are separated. However, the quantitative relations of

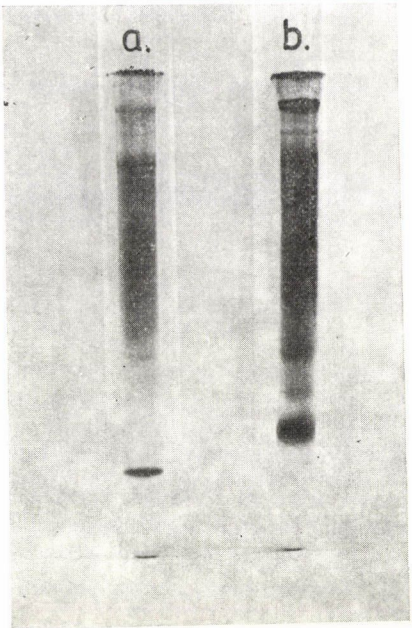

Fig. 6. Electropherogram of carrots and parsley root
a) carrot
b) parsley root

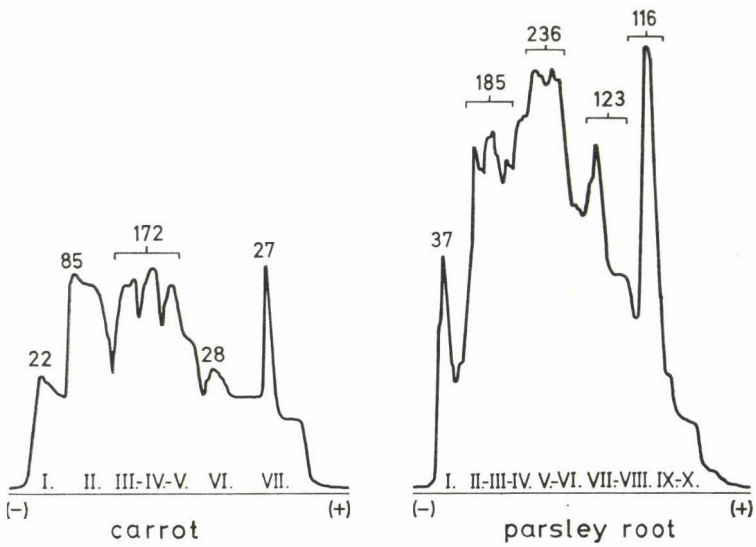

Fig. 7. Densitograms belonging to carrot and parsley root

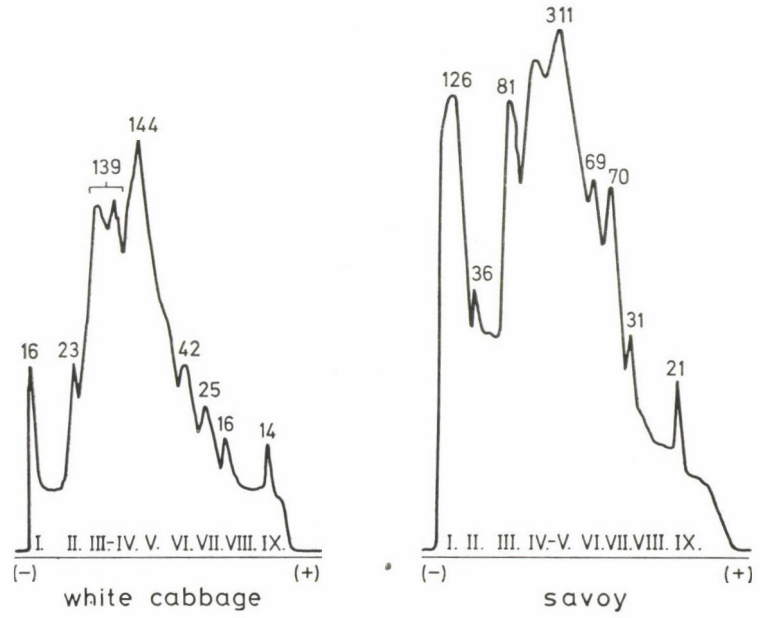

Fig. 8. Densitograms of white cabbage and savoy (Figures refer to the size of the area)

individual fractions, as seen in the densitograms, show a significant difference. This is reflected in the values given in Table 10. In the case of savoy, the least motile fraction (marked I) is substantially more intense and a similar difference, though less marked can be observed with fraction VII.

2.2.3. Quantitative changes in soluble proteins during storage. It is a well-known fact that plant particles removed from their stem undergo an increased dissimilation process. Protein hydrolysis is one of these processes and this affects strongly the food value of the vegetable and its storage life.

Table 10

Numerical evaluation of the densitograms belonging to white cabbage and savoy

		Protein fractions									
		I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	Σ
White cabbage	I.V.[a]	16	23	139		144	42	25	16	14	419
	%[b]	3.8	5.5	33.2		34.4	10.0	6.0	3.8	3.3	100
Savoy	I.V.	126	36	81	311		69	70	31	21	745
	%	16.9	4.8	10.9	41.7		9.3	9.4	4.2	2.8	100

[a] I. V. = Area below the light absorption peaks enclosed with the base line
[b] % = The area under individual peaks as percentage of the total area

Spinach (*Spinacia oleracea* cv. Matador), a vegetable of short and intense storage life, was chosen for the experiments. The spinach was freshly picked and stored at room temperature (*cca* 20 °C). Pressed juice was prepared on three consecutive days.

Changes in the soluble protein content and in the proportions of the fractions are illustrated in Fig. 9 and the values are summarized in Table 11.

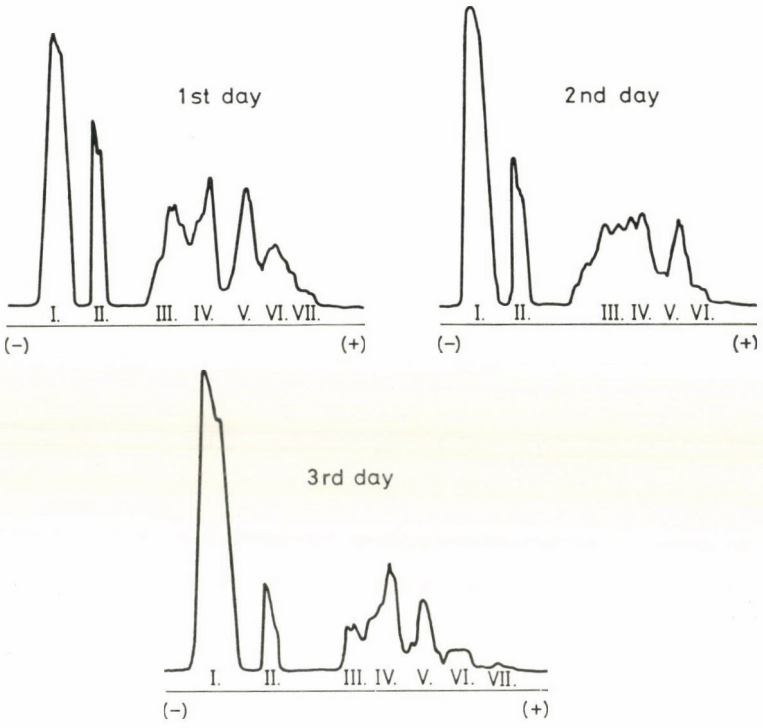

Fig. 9. Soluble protein fractions in spinach as affected by three days storage

As it may be seen in the electropherogram the soluble protein of spinach separated into 7 fractions. Characteristic are the two fractions of low motility (marked I and II) remaining near the upward zone. Reduction in the amount of the total soluble protein content is characteristic of changes during storage. The loss was 8.7% on the second day and 5.7% on the third day. This change – consistent in its tendency and similar in numerical value in repeated experiments – seems suitable to characterize the physiological changes occurring in soluble proteins.

The reduction in the soluble protein content did not bring about a general reduction of the individual protein fractions, in fraction I definite increase was observed. At the same time, the percentage proportions shifted substanti-

Table 11

Total soluble protein content and changes in the individual fractions during storage of spinach

Storage time (day)	Total	Soluble protein fractions					
		I.	II.	III.–IV.	V.	VI.	VII.
		Integrated unit					
1	255	86	30	77 (36+41)	33	25	4
2	233	100	26	80	23	44	–
3	219	126	15	48 (10+38)	20	8	2
		Percentage value					
1	100	33.7	11.7	30.2 (14.1+16.1)	12.9	9.8	1.5
2	100	42.9	11.2	34.3	9.9	1.7	–
3	100	57.5	6.8	22.0 (4.6+17.4)	9.1	3.9	0.9

ally and the boundaries between the fractions became indistinct. These phenomena could be unambiguously evaluated by further plant biochemical and biological investigations into the size and charge of the proteins.

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IDENTIFICATION OF A NEW AMINE IN ORANGE FLAVOUR FROM AQUEOUS CONDENSATE OBTAINED DURING THE CONCENTRATION OF THE JUICE

J. PINO

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An analytical procedure is described for the isolation and identification of trace-level amines from an aqueous orange condensate obtained during the industrial concentration of the juice. Amines were separated as salts by acid extraction from aqueous condensate and regenerated for the gas-chromatographic analysis. Five amines were isolated. One of them, ethyl o-aminobenzoate (ethyl anthranilate) is a newly identified citrus constituent.

In recent years, the use of chromatographic and spectroscopic techniques has greatly accelerated the identification of specific flavour-producing compounds in fruit and fruit products. Several investigators have studied the oxygen-containing flavour components of citrus juices and essential oils in order to assess the importance of this component group (IKEDA *et al.*, 1962; ATTAWAY *et al.*, 1962, 1964; TERANISHI *et al.*, 1966). However, no data on aliphatic or aromatic amines could be found in the literature except the paper by WOLFORD and co-workers (1962). These authors used gas chromatography for the tentative identification of methyl N-methyl anthranilate in aqueous orange essence. However, nitrogen-containing compounds also play an important role in the taste and odour of certain fruits and fruit products (ASKAR *et al.*, 1972; ASKAR, 1973; UDENFRIEND *et al.*, 1959). Therefore, it could be expected that they are important flavour-producing compounds in orange juice as well.

This paper reports the isolation and identification of slightly volatile amines from an aqueous orange condensate obtained during the industrial concentration of the juice.

1. Materials and methods

1.1. Isolation of the volatile amines from aqueous orange condensate

Aqueous condensate obtained during the concentration of orange (*Citrus sinensis* L. Osbeck) juice in a local factory was used in the experiments.

To 6 000 cm^3 of aqueous condensate, hydrochloric acid was added to obtain pH 1. The acidic solution was concentrated to 250 cm^3 in a rotary vacuum

evaporator. The acid residue was subjected to steam distillation in order to eliminate the remaining non-acidic volatile compounds. After the collection of 500 cm^3 of distillate, the residue was cooled in an ice bath and carefully neutralized with 10% (w/w) sodium hydroxide solution. Then, the volatile amines were extracted with ether (3 times, 50 cm^3 each). The organic layer was washed with water, dried with anhydrous sodium sulphate and carefully concentrated to 0.5 cm^3 for further chromatographic analysis.

Since the concentration of all other volatile components was high in the aqueous condensate the isolation of amines for further gas-chromatographic analysis presented a serious problem. The procedure described above is not suitable for highly volatile amines. However, slightly volatile amines could be successfully isolated in trace amounts.

1.2. Conditions of gas chromatography and spectroscopic techniques

Gas-chromatographic analysis was carried out on a *Packard–Becker* Model 419 equipped with a flame ionization detector and a 3 m long, 0.4 cm o.d., stainless steel column packed with 60/80 mesh *Chromosorb G* (acid washed) coated with 5% polyethylenglycol adipate (BDH, England). The GC conditions were as follows: carrier gas: argon, flow rate: 25 $cm^3\,min^{-1}$, injector temperature: 250 °C, detector temperature: 250 °C. Optimum separations were obtained when the column temperature was programmed from 80 °C to 200 °C at a rate of 5 °C min^{-1}.

Preparative gas-chromatographic analysis was carried out on a *Pye Unicam* Model 105 equipped with a flame ionization detector and 8 m long 0.9 cm o.d. glass column packed with 30/60 mesh *Chromosorb* W coated with 15% *Carbowax* 20 *M* (BDH, England). The column temperature was programmed from 100 °C to 200 °C at 5 °C min^{-1}.

The gas chromatographic separation of the individual amines presents some difficulties (MADARASSY-MERSICH *et al.*, 1978). The conditions used gave good results for the separation of the slightly volatile amines.

The infrared spectrum of the compound collected after GC separation was obtained with a *Carl Zeiss* IR-20 instrument. The NMR spectrum was obtained using a *Hitachi* instrument at 60 MHz and the mass spectrum was recorded with a *Hitachi* RMU-6D system.

2. Results

A typical gas chromatogram of the separated amines is shown in Fig. 1. The first three and the last components had relatively minor peaks and could not be condensed for further study. Component 4 was present in sub-

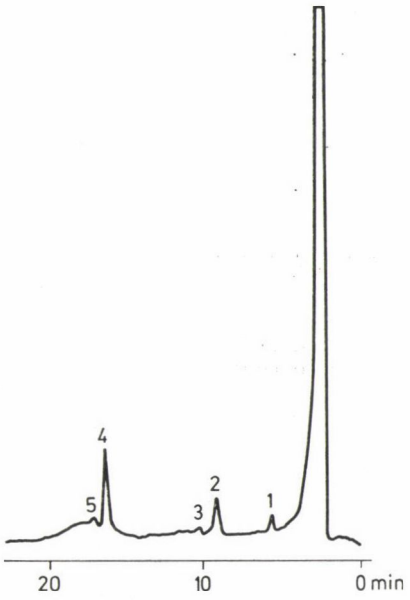

Fig. 1. Gas-chromatographic separation of amines isolated from an aqueous condensate obtained during the concentration of orange juice on a polyethyleneglycol adipate column. Injector temperature: 250 °C; detector temperature: 250 °C; Carrier gas flow rate: 25 cm^3 min^{-1}. Temperature program from 80 °C to 200 °C at 5 °C min^{-1}

stantial quantity and gave a prominent peak. It was condensed and studied through its infrared, NMR and mass spectrum.

The infrared spectrum of the isolated compound (Fig. 2) shows two strong bands at 3480 and 3370 cm^{-1} corresponding to the $-NH_2$ stretching vibrations of aromatic primary amines. The bands at 2970 and 2940 cm^{-1} indicate the presence of methyl and methylene groups resp., and the band at 1700 cm^{-1} indicates the presence of a conjugated carbonyl group. Bands at 1620, 1590 and 1580 cm^{-1} confirm the presence of an aromatic ring in the isolated compound. The bands at 1250 and 1110 cm^{-1} are typical of a benzoate ester. The bands at 760 and 710 cm^{-1} are the bending vibrations assigned to an ortho-disubstituted benzene ring.

The NMR spectrum of the isolated compound is shown in Fig. 3. The triplette at 1.36 ppm indicates the presence of 3 protons in a methyl group attached to a methylene group. The quartette at 4.28 ppm is due to 2 protons in a methylene group attached to a methyl group. Since this quartette occurs at a lower field it can be assigned to the structural feature $-CO-O-CH_2-$. The signal at 5.66 ppm indicates the presence of two protons in an amine group. The multiplets at 6.60 ppm (H_B and H_D), 7.15 ppm (H_C) and 7.84 ppm (H_A) confirm the presence of an ortho-disubstituted benzene ring.

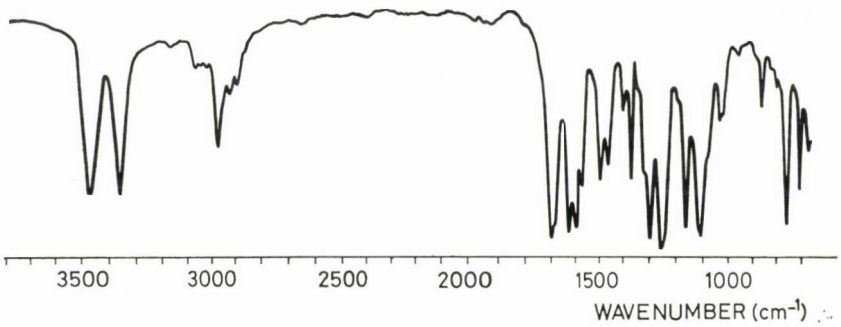

Fig. 2. Infrared spectrum of the amine isolated from an aqueous condensate obtained during the concentration of orange juice

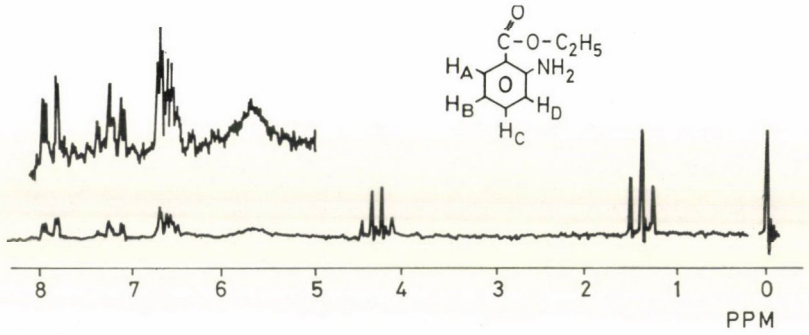

Fig. 3. Nuclear magnetic resonance spectrum of the amine isolated from an aqueous condensate obtained during the concentration of orange juice

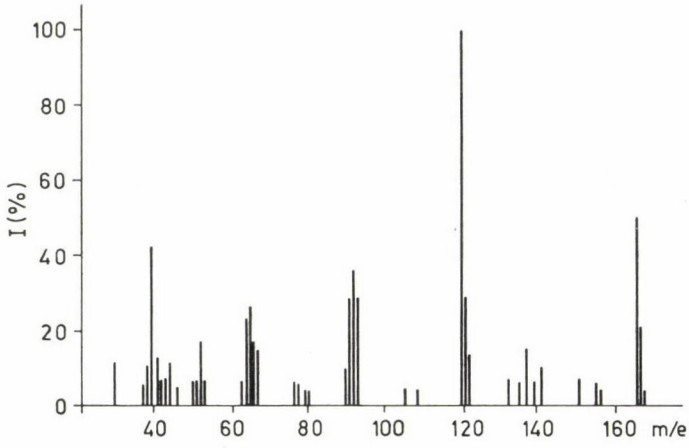

Fig. 4. Mass spectrum of the amine isolated from an aqueous condensate obtained during the concentration of orange juice

The mass spectrum of the isolated amine (Fig. 4) finally confirms the presence of an ethyl benzoate skeleton and an amine group (molecular peak at 165 mass units). The base peak at 119 mass units is due to the "ortho elimination" of ethanol (BUDZIKIEWICZ *et al.*, 1967).

The data obtained from the spectra indicate that the compound isolated is ethyl anthranilate, a newly identified citrus constituent.

3. Conclusions

The study presents an analytical procedure for the isolation of trace-level, slightly volatile amines from aqueous orange condensates obtained during the industrial concentration of orange juice.

One of the amines separated, ethyl anthranilate, has not been previously reported as a constituent of orange or other citrus fruits.

Obviously, the ultimate goal of such an investigation is the sensory evaluation of the identified constituent as to its contribution to the aroma and flavour of orange juices. The examination of the organoleptic properties of the compound identified in this work and the identification of the other unknown amines are in progress.

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DETERMINATION OF BENZO/A/PYRENE IN CIGARETTE SMOKE

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Polycondensed aromatic hydrocarbons (PAH) are physiologically active components of cigarette smoke. The classic representative of this group and, at the same time one of the physiologically most active compounds is benzo/a/pyrene.

It has been detected almost everywhere in our environment and is used to indicate the presence of PAH compounds.

In this paper a spectrophotometric method for the determination of benzo /a/pyrene based on ultraviolet absorption is described. A difficulty in the determination of this compound lies in the fact that it is present in smoke only in minute amounts (some ng per cigarette) and it is essential to separate it from the great number of other compounds present in cigarette smoke. A multi-step purification and separation procedure, based on combination of steps of the methods found in related literature, was developed.

The method described, within the reproducibility and recovery limits, is suitable for the quantitative determination of benzo/a/pyrene in the smoke of different cigarettes.

As far as it is known at present, cigarette smoke consists of over 1 200 components. The major part is formed by four components (90%): N_2, O_2, CO_2, H_2. The other components represent only 10%.

Although several of the latter components are present only in trace amount, their knowledge is nevertheless important because of their physiological effect.

Carcinogenic compounds form part of the biologically active components of smoke, the best known of which are the polycondensed aromatic hydrocarbons (PAH). The biological effect of these compounds has been known since the pioneering work of COOK and co-workers (1933). They have shown in the early thirties that for the carcinogenic effect of coal-tar this group of compounds and particularly its most active representative, benzo/a/pyrene is responsible.

It has been shown later that PAH compounds occur in our environment everywhere, with the advance of urbanization and the use of chemicals in ever increasing amounts (KERTÉSZ-SÁRINGER & MORLIN, 1972; BUTLER, 1975). They were detected in water (SCHWARZ & WASIK, 1976), in tar and petroleum derivatives (SOÓS, 1972) even in food items (coffee, bread, fish *etc.*), particularly in smoked and roasted goods (SOÓS & FŐZI, 1974; SOÓS, 1974; STEINIG, 1976).

More intense research into the polycondensed aromatic hydrocarbons started in the second half of the fifties awakening the interest of medical science and tobacco industry experts. In experiments carried out on animals, it was attempted to find a correlation between the carcinogenic effect and chemical structure and to develop appropriate methods fort he detection and determination of individual components of this group of compounds.

The most characteristic representatives of polycondensed aromatic hydrocarbons occurring in cigarette smoke classified according to their biological activity are shown, on the basis of the work of WYNDER and HOFFMANN (1967), in Table 1.

Table 1

Polycondensed aromatic hydrocarbons most frequently occurring in cigarette smoke

(WYNDER & HOFFMANN, 1967)

Carcinogenic PAH compounds	Relative carcinogenic activity	Quantity (μg per 100 cigarettes)	Non-carcinogenic PAH compounds	Quantity (μg per 100 cigarettes)
Benzo/a/pyrene (3,4-benzpyrene)	+++	2.5	Pyrene	7.5–10.5
Dibenz/a, h/anthracene	+++	0.4	Fluoranthene	7.5–10.5
Benzo/b/fluoranthene	++	0.3	Methylchrysene	7.6
Benzo/j/fluoranthene	++	0.6	Dimethylchrysene	10.3
Dibenzo/a, l/pyrene	++	in traces	11-/1-benzo/a/fluorene	4.1
Benz/a/anthracene	+	0.3	Methyl-11-H-benzo/a/fluorene	3.5
Chrysene	+	6.0	Benz/g, h, i/-fluoranthene	0.3
Benzo/e/pyrene	+	0.3	Benz/k/fluoranthene	0.7
Indeno/1,2,3-cd/pyrene	+	0.4	Perylene	0.3
			Benz/g,h,i/-perylene	0.6
			Anthantrene	0.3

It may be seen in Table 1 that of the carcinogenic substances present in smoke benzo/a/pyrene is one of the most active compounds, thus is most harmful to human health.

PAH compounds are formed during the burning of tobacco. Two mechanisms of formation are assumed (BADGER *et al.*, 1965; HOFFMANN & WYNDER, 1972).

According to the first assumption, the condensed rings are formed by the pyrolytic formation of free hydrocarbon radicals and their combination. Formation of these compounds is shown in Fig. 1.

According to the second assumption, in the course of *Diels-Alder* addition of dienes into dienofils, new rings are formed and these are dehydrogenated into polycondensed aromatic systems. This mechanism provides an explana-

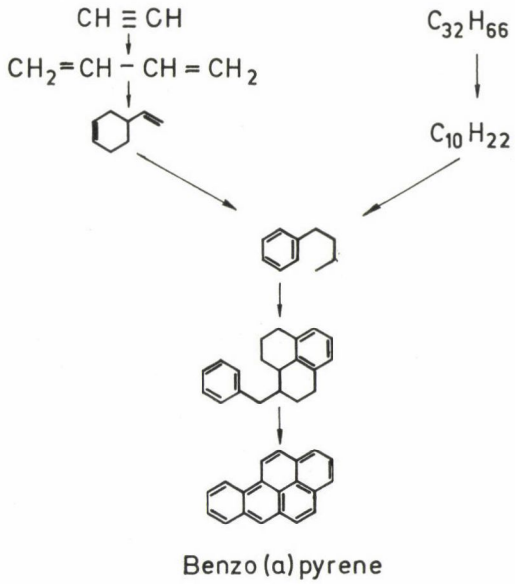

Fig. 1. An assumed formation mechanism of condensed cyclic compounds

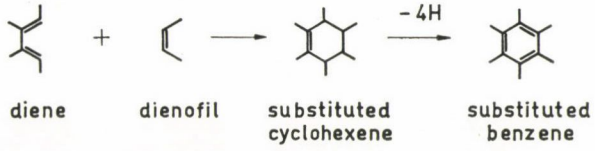

Fig. 2. Second assumed formation mechanism of condensed cyclic compounds

tion for the relatively high concentration in smoke of alkylated PAH derivatives. This way of formation is shown in Fig. 2.

The diene present in smoke in the largest quantity (0.3–0.7 mg per cigarette) is isoprene. Volatile dienes and dienofils are formed during the pyrolysis of tobacco terpenes and form about 0.2–2.0% of the dry leaves.

Many researchers were engaged in the analysis of PAH compounds, because their detection and quantitative determination is the first step in their biological investigation and in the development of products of lower PAH content which will be less harmful to human health.

The main difficulty of their determination lies in the small quantity in which they are found in smoke (some ng per cigarette) while in order to be able to determine them they must be separated from a great number of other components. A further problem is the separation of the PAH compounds from one another because their properties are very similar. Thus the majority of investigations was aimed at the determination of benzo/a/pyrene only. However,

recently LEE and co-workers (1976) succeeded in separating 143 different polycondensed aromatic hydrocarbons and identifying them.

Since the analytical determination of benzo/a/pyrene is the most advanced, its biological effect is the most dangerous and it may be used for the indication of the presence of other polycondensed aromatic hydrocarbons, it seemed desirable to develop a method for its quantitative determination (PRIOTSCH *et al.*, 1978).

1. Materials and methods

The smoke condensate of experimental cigarettes, made of tobacco *Hevesi* was used as test material.

Cigarettes were smoked on a special machine (*Filtrona* type 300) automatically, at parameters specified in the international standard (CORESTA STANDARD METHOD, 1968).

In order to be able to collect the smoke condensate without loss, a trap containing glass rings and cooled by a mixture of acetone and dry-ice was used. For each analysis 200 cigarettes were machine-smoked.

The collected condensate was processed according to the scheme given below by liquid-liquid extraction, separation and purification by thin-layer chromatography:

- Elution of the collected condensate with MeOH-ether (1:4) mixture, 100 cm^3 per trap
- Measurement of the weight of the evaporated condensate. Dissolved in the solvent mixture MeOH-ether (1:4)
- Preparation of the neutral fraction. Extraction:
  2×80 cm^3 1 *N* H_2SO_4
  2×80 cm^3 1 *N* NaOH
  3×100 cm^3 distilled water
- Evaporation. Residue dissolved in 100 cm^3 MeOH-H_2O (9:1) solvent mixture
- Hydrocarbon fraction: extraction by 2×100 and 2×50 cyclohexane
- Evaporation. Residue dissolved in 80 cm^3 cyclohexane
- Enhancement of the polycondensed aromatic hydrocarbon content: Extraction with 3×50 cm^3 nitromethane
- Evaporation of the nitromethane fraction. Residue dissolved in 2 cm^3 $CHCl_3$
- Chromatography on silicagel thin-layer plates. Repeated development in 4:1 mixture of cyclohexane–benzene
- Separation of the fluorescent zone. Elution in 50 cm^3 benzene by shaking. Evaporation, residue dissolved in 2 cm^3 of ether

- Chromatography on cellulose acetate thin-layer plates. Solvent: EtOH–toluene–H_2O (17:4:4) mixture
- Separation of the benzo/a/pyrene fraction. Elution with 20 cm^3 of benzene, evaporation
- Measurement of the UV absorption of benzo/a/pyrene at 385 nm. For measurement residue dissolved in 5 cm^3 of cyclohexane
- Quantitative evaluation, based on the calibration curve of standard substance.

The absorption maximum of benzo/a/pyrene at 385 nm is suitable for quantitative evaluation because at this wavelength optical density is linearly correlated with concentration and the other components have no absorption maximum at this point.

Figure 3 shows both the spectra of the standard benzo/a/pyrene (solid line) and of the benzo/a/pyrene obtained from cigarette smoke (dashed line).

The concord of peaks used for identification is well visible. The quantitative determination was carried out with the calibration curve obtained from the series of solutions between 0.05 μg and 5.0 $\mu g\ cm^{-3}$ of the standard substance ($r = 0.998$). Equation of the line:

$$Y = 1.27 \cdot 10^{-1} X + 3.3 \cdot 10^{-4}$$

where Y = the measured extinction value
X = the amount of benzo/a/pyrene ($\mu g\ cm^{-3}$)

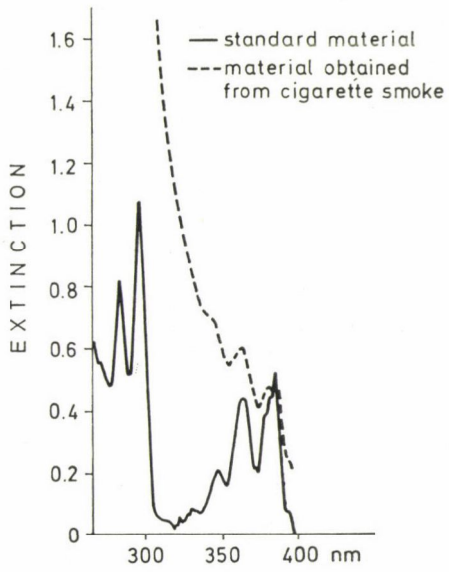

Fig. 3. Spectrum of benzo/a/pyrene

2. Results and conclusions

Of the methods used for the determination of benzo/a/pyrene, the above method proved to be the most satisfactory in our laboratory. This was developed after the critical evaluation of methods found in the literature by their combination and appropriate modification (FÜGEDI-NÉMETI, 1977).

To control the reliability of the method, first it was tested for the recovery of standard substance. It is known from the literature that the average benzo /a/pyrene content of cigarettes amounts to 20–25 ng. Accounting for this, the process of purification was carried out with an amount of substance corresponding to the upper and lower limit of the range, related to 200 cigarettes. Thus, 10 μg and 4 μg of benzo/a/pyrene resp., were processed by the method as described above. Results are summarized in Table 2.

Table 2

Recovery of benzo/a/pyrene in the absence of smoke condensate

Compound weighed (μg)	Optical density prior to purification	Optical density after purification	Benzo/a/pyrene recovery (μg)	Percentage of substance recovered (%)
	at 385 nm			
10	0.260	0.250	9.83	98.3
10	0.260	0.240	9.43	94.3
10	0.260	0.245	9.63	96.3
4.12	0.105	0.095	3.72	90.3
4.12	0.105	0.092	3.60	87.6
4.12	0.105	0.095	3.72	90.3
Averages of 3 parallel measurements				
10	0.260	0.245	9.63	96.3
4.12	0.105	0.094	3.68	89.4

As it may be seen, recovery amounted to 94–98% or 88–90% of the original. If the standard substance was added to the condensate of about 200 cigarettes, recovery amounted to 74–80%, as it is shown in Table 3. This shows good agreement with data found in the literature (GRIMMER, 1961; DAVIS *et al.*, 1966; MÜLLER *et al.*, 1967; OAKLEY *et al.*, 1972).

This lower recovery may be explained by the intricacy of the smoke condensate and the chemical properties of the substance.

Table 3

Recovery of benzo/a/pyrene added to smoke condensate

Benzo/a/pyrene (μg)		Substance recovered	
added	theoretically expected	(μg)	(%)
—	12.58 (initial amount)		
4	16.58	12.78	77.08
6	18.58	13.76	74.06
8	20.58	16.52	80.27
10	22.58	17.70	78.39
Average of recovered percentages			77.45
Range of recovered percentages			74–80

Further, the reproducibility of the method was established. Results are shown in Table 4.

Table 4

Reproducibility of the method

Number of test	Optical density measured (E_{385} nm)	Quantity found (μg)	Benzo/a/pyrene content (ng per cigarette)
1	0.500	11.80	64.85
2	0.640	15.10	75.54
3	0.520	12.27	61.35
4	0.590	13.92	69.64
5	0.690	16.29	81.45
Averages and deviation	0.590	13.88	70.57±8.09

The corrected deviation as established from the data in the Table is ±8.09 ng per cigarette. Percentage error inherent in the method is 11.5%. Thus the method above described, beside being reliable and of good recovery, is suitable for the quantitative determination of benzo/a/pyrene occurring in different kinds of cigarettes.

The authors consider the method to be particularly suitable for establishing the efficiency of filters of various types aimed at reducing the harmful components of cigarette smoke.

Since, however, the other PAH components are also harmful to health it is not sufficient to be able to determine one carcinogenic compound, even

if the most active one, because they may intensify their mutual effect. Thus, efforts are made in this Institute to determine the PAH compounds individually by applying more sensitive gas-chromatographic methods.

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APPLICATION OF ION-SELECTIVE MEMBRANE ELECTRODES IN TOBACCO ANALYSIS

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A review is given of the methods used in recent years to determine the volatile components of tobacco smoke (HCN, H_2S). These methods involve the use of an automatic titrimeter and ion-selective membrane electrode. Error inherent in the method: ± 2.0 or $\pm 1.0\%$; recovery: 94.6–96.1 or 92.0–98.4%. The methods are suitable for the analysis of a great number of samples, thereby providing a data basis suitable for the representative evaluation of products commercially available and for the exploration of relations enabling the development of tobacco products of milder physiological effect.

Health aspects of smoking are followed with interest all over the world. The aim of the tobacco industry is to maintain the hedonic character of smoking while reducing its deleterious effects.

For the deleterious effects the physiologically active components of smoke are responsible. These are chemically diverse and are present in the particle and gas phase of smoke in various amounts.

The task of product development is to produce goods of milder physiological effect. In order to be able to investigate the possibilities for reducing the harmful effects of smoke and/or to establish relations useful from the aspect of health, the physiologically active components of smoke, and their quantity has to be known.

To analyse the particle phase of smoke, the standard methods of CORESTA (Cooperation pour les Recherches Scientifiques relatives au Tabac) are mainly used. To determine the volatile components of the gas phase, new methods have been developed in recent years. In selecting the methods the aim was that they should be suitable for the analysis of a great number of samples by automatic procedures so that the data obtained by repeated analyses could be evaluated by methods of mathematical statistics. The present study gives an account on procedures for the determination of hydrogen cyanide and hydrogen sulfide in cigarette smoke carried out with an automatic titrimeter and ion-selective electrodes.

1. Materials and methods

1.1. Determination of hydrogen cyanide in cigarette smoke

The hydrogen cyanide content of smoke is determined with $AgNO_3$ solution by potentiometric titration using ion-selective membrane electrodes and automatic final point signalling.

1.1.1. Principle of the method. CN^- ions react with Ag^+ ions according to the following equilibrium reaction:

$$Ag^+ - 2CN^- \rightleftharpoons [Ag(CN)_2]^- \rightarrow AgCN$$

A cyanide ion-selective electrode is used, thereby eliminating the disturbing effects of other ions or organic compounds and the point of equivalence is well defined.

1.1.2. Procedure

1.1.2.1. Preparation – Cigarettes selected and conditioned according to weight and resistance to air were mechanically smoked according to CORESTA standard No. 10. A 100-cm³ *Drechsel* gas washing flask containing 60 cm³ 2% NaOH solution is attached behind the *Cambridge* filter to absorb the HCN content of smoke. Five cigarettes are smoked for each determination. On finishing smoking the washing flask is washed through with 60 cm³ 2% NaOH solution and in the 120 cm³ solution obtained the Cambridge filter is also put in. Prior to determination the solution is filtered through a wad. The first part of the filtrate is discarded and aliquots of 20 cm³ are used for titration. The 20 cm³ solution is filled up to 60 cm³ with distilled water before titration.

1.1.2.2. Reagents – 2% NaOH solution
0.001 *N* $AgNO_3$ solution
0.1 *N* KNO_3 solution
(for the reference electrode)

1.1.2.3. Establishment of the point of equivalence. – The point of equivalence is established by manual titration in at least five parallel measurements. Electrode chain: CN^- ion-selective membrane electrode (*Radelkis* OP–CN 7112 d) – inert salt-bridge reference electrode (*Radelkis* OP 8212). The amount of the titrating solution (cm³) is plotted against mV and the point of equivalence is established from the potentiometric curve by graphic technique. In further studies this point of equivalence is adjusted by the automatic titrimeter (Fig. 1).

1.1.2.4. Measurement – Measurements are carried out with an automatic titrimeter (*Radelkis* OP 506) by the electrode chain: CN^- ion-selective mem-

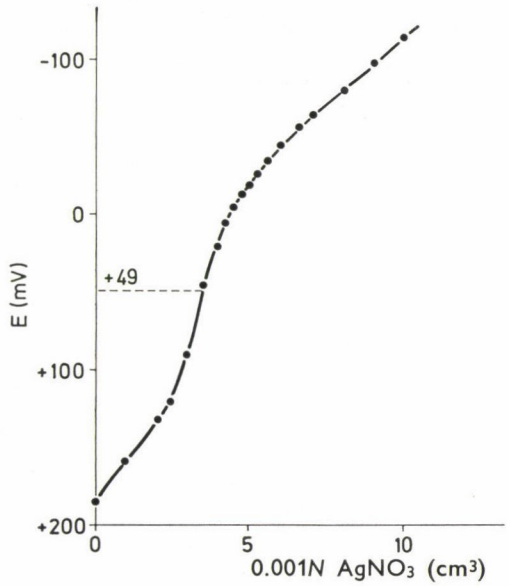

Fig. 1. Potentiometric titration curve of HCN

brane electrode (*Radelkis* OP–CN 7112 D) – inert salt-bridge reference electrode (*Radelkis*, OP 8212).

Point of equivalence: +49 mV, preperception: −80 mV, time delay: 5 s. The automatic titrimeter stops the inflow of the measuring solution at the point of equivalence and the amount of the solution used can be read.

1.1.2.5. Calculation – Results are given in μg HCN per cigarette:

$$\text{HCN } \mu g \text{ per cigarette} = V_{cm^3} \cdot 64.8$$

where:

$$V_{cm^3} = \text{amount of the } 0.001\,N \text{ } AgNO_3$$

measuring solution in cm^3.

1.2. Determination of hydrogen sulfide

H_2S is determined in the smoke with $AgNO_3$ solution by potentiometric titration, using ion-selective membrane electrodes and automatic end point signalling.

1.2.1. Principle of the method. Ions S^{2-} and Ag^+ react according to the following equilibrium reaction:

$$2Ag^+ + S^{2-} \rightleftarrows [AgS]^- \rightarrow Ag_2S$$

The sulfide ion-selective membrane electrode eliminates disturbing effects and the point of equivalence is well defined.

1.2.2. Procedure

1.2.2.1. Preparation – As described in relation to the HCN content.

1.2.2.2. Reagents – As described in relation to the HCN content.

1.2.2.3. Determination of the point of equivalence – The point of equivalence is determined by manual titration in at least 5 parallel measurements. Electrode chain: S^{2-} ion-selective membrane electrode (*Radelkis* OP–S 7111 D) – inert salt-bridge reference electrode (*Radelkis* OP 8212). The volume of the measuring solution in cm^3 is plotted against mV and the point of equivalence is determined from the curve graphically. In further determinations this point of equivalence is adjusted by the automatic titrimeter (Fig. 2).

1.2.2.4. Measurement – Measurements are carried out with the automatic titrimeter (*Radelkis* OP 506) on the electrode chain: S^2 ion-selective membrane electrode (*Radelkis* OP–S 7111 D) – inert salt-bridge reference electrode (*Radelkis* OP 8212). Point of equivalence: +175 mV, preperception: −300 mV, time delay: 5 s. The automatic titrimeter stops the inflow of the measuring solution at the point of equivalence and the volume used is read on the burette.

1.2.2.5. Calculation – Results are given in μg H_2S per cigarette:

$$H_2S\ \mu g\ \text{per cigarette} = V_{cm^3} \cdot 20.4$$

where:

$$V_{cm^3} = \text{volume of } 0.001\ N\ AgNO_3$$

solution used in cm^3.

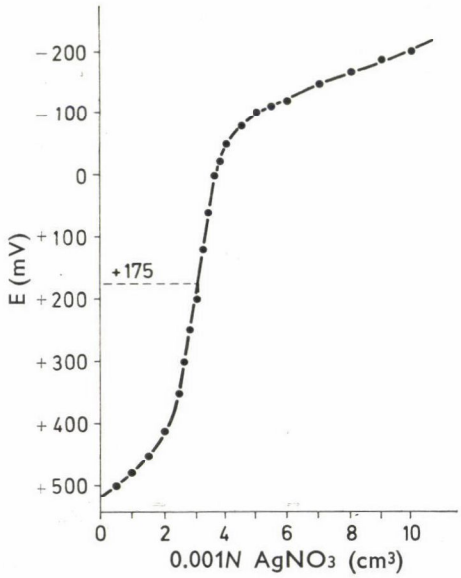

Fig. 2. Potentiometric titration curve of H_2S

1.3. Error of the method, recovery

Table 1 contains data on the error and recovery in both methods.

Table 1

Characteristic data of the determination methods

Method	Average error of one measurement, (%)	Recovery, (%)
HCN	±2.0	94.6–96.1
H_2S	±1.0	92.0±98.4

2. Results and conclusions

The methods described above may be used routinely. It is necessary to carry out a great number of determinations in order to test commercial products and for product development.

Table 2

Informative data on some products on the market

Products with smoke filter	HCN μg per cigarette	H_2S μg per cigarette
Románc	0.40	82.3
Fecske	0.43	70.7
Symphonia	0.40	107.1
Sophianae	0.33	60.3
Délibáb	0.11	62.9
Helikon	0.28	64.6

Data for HCN are averages of at least 18 measurements and for H_2S of at least 3 measurements.

In relation to product development a wide range of investigations was made to test various types of filters and filter-additives to establish their efficiency:

$$H\% = \frac{(F_N - F) \cdot 100}{F_N}$$

where:

$H\%$ = filter efficiency, %
F_N = determined amount of compound per cigarette without filter
F = determined amount of compound per cigarette with filter

Recently it has become known that by diluting cigarette smoke – by applying high-porosity cigarette paper, perforations either on the filter or on the paper itself – the amount of the gase-phase components of smoke can be substantially reduced. An important part of the research work of the author extends over this problem.

Earlier the HCN content was determined by the titrometric technique of CIGARETTE COMPONENTS Ltd. (1964). However, this method proved to be time-consuming and the colour change, due to the original colour of the samples, was difficult to establish. To determine the H_2S content, the spectrophotometric method of CARUGNO (1961) was tested.

The H_2S and HCN determination methods were developed on the basis of the papers of MATTINA (1972), REIF (1972) VICKORY and GAUNT (1972). The method was somewhat modified in order to make preparatory work identical for both determinations.

As it may be seen from the above, our aim in selecting the method was to find rapid, simple and accurate automatic techniques requiring identical preparation and suitable for our purposes.

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STUDY OF PUROTHIONIN ANALOGUES OF CERTAIN CEREALS

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In this study the protein-like properties of purothionin (fraction distribution, amino acid composition) and qualitative and quantitative parameters of lipids forming complexes with it are compared to those of the analogues obtained from barley, rye and oat.

The isolation methodology of purothionin (splitting with hydrochloric acid the petroleum ether extract in a moisture-free medium) applied to the extracts of the above grains, resulted in all three cases in the production of a protein analogous with purothionin. For rye and oat this is in contradiction with data found in the literature: from rye an analogue was separated by a roundabout rout, while no purothionin analogue had been found in oat.

We succeeded in every case in isolating from petroleum ether extract the more stable core of the thionin-lipid complex dominantly containing polar lipids (lipopurothionin analogues). Thus it was possible to compare the thionins and the lipid complexes of these four cereals.

According to the results the individual representatives of the thionin family are very similar in structure. In consequence, the measure of their lipid binding capacity, the kind and proportion of bound lipids and their toxicity to yeasts was found very similar, too.

Purothionin, or to be more exact, its two low-molecular fractions marked α and β belong to the most well-known proteins of non-enzymic character in the plant world. The amino acid sequence of purothionin was reported by two independent research teams (MAK & JONES, 1976; JONES & MAK, 1977 and OHTANI *et al.*, 1975). Its structure and biological function was discussed in more than 30 papers since the publication of the first report on the subject by BALLS and HALE (1940). Several reviews have been published on the purothionin literature the latest of which is the work of POMERANZ (1976). In a previous paper, the complete bibliography of this subject has been published (BÉKÉS & SMIED, 1980).

Although much effort was spent on isolating proteins of purothionin character from other grains, till recently this met with success only in the case of barley. The first to describe this substance, called by the authors hordothionin, were REDMAN and FISHER (1969). Hordothionin may be considered unambiguously a purothionin analogue. HOSENEY and co-workers (1971) and OKADA and YOSHIZUMI (1973) discovered that beside the similarity in the protein structure or in consequence of this, the lipid binding capacity and the toxic character resemble also that of purothionin.

The knowledge of the amino acid sequence of purothionin enabled the emergence of a new approach in the research of the thionin family. It came to light, namely, that the oak, a tree taxonomically very far removed from wheat, contained a protein of an amino acid sequence very similar to that of purothionin (MAK & JONES, 1976). The existence of this protein, called viscotoxin, raises the possibility of the general presence in the plant world of this protein of characteristically high basic amino acid and cystine content of a given function. According to MAK and JONES (1976), the fact that at the time of the discussion of this question no other thionins beside purothionin and hordothionin were known, can be explained by the low quantity of the substances representing certain components of the lipid complexes of thionins, which may limit in some plant species the solubility in petroleum ether, impeding thereby the solubilization of the protein in organic solvents. Following this train of thoughts, HERNANDEZ-LUCAS and co-workers (1977) succeeded in identifying the limiting factor as digalactosyl diglyceride. Subsequently they isolated indirectly the purothionin analogue of rye and named it secalethionin (HERNANDEZ-LUCAS *et al.*, 1978. The "thionin family" concept was used the first time in this paper.) This discovery permitted the formation of a uniform picture of the homologous chromosomal localization of the thionin gene in different wheat and rye varieties and the difference in the α and β ratio of fractions in *T. durum*, *T. aestivum* and *Triticale* was explained (MONGE *et al.*, 1979). In contrast to the detailed knowledge of the protein structure of purothionin the knowledge of the purothionin-lipid complex and particularly of the biological, biochemical functions of the protein and its complex is substantially more modest. From the research work carried out at our Department over several years a mass of partial knowledge of the complex isolated from the petroleum ether extract of wheat accumulated (LÁSZTITY *et al.*, 1978, 1979a, b). On the basis of the comparison of complexes gained under different conditions and of model experiments the structural model of the lipid complexes of purothionin was outlined in a previous paper (BÉKÉS & SMIED, 1980). It was established that the relatively stable inner complex represented by phospholipids in salt-like bond of varied strength and by glycolipids in interrelation with them, is accompanied by a neutral lipid region stabilized by hydrophobic bond (BÉKÉS & SMIED, 1980). Information on lipids bound in complex by other grain thionins is available only from the short report of HOSENEY and co-workers (1971).

The functions of purothionin in plant physiology are practically unknown. A significant change in the quantity of the protein fraction could not be proved during ripening or germination (BÉKÉS, 1980). The few data found in the literature on the enzyme-inhibitory character of purothionin are not convincing: the trypsin and chymotrypsin inhibitory functions have been disproved and papain inhibition was not confirmed (POMERANZ, 1976; MONGE *et al.*, 1979).

On the other hand it has been known for long (COULSON *et al.*, 1942; BALLS & HALE, 1944) and confirmed by several authors (FERNANDEZ DE CALAYA *et al.*, 1972; OKADA *et al.*, 1970a, b) that purothionin and hordothionin are active against yeasts and have a toxic effect on some bacteria while exerting a contractive effect on the uterus of mammals.

In the present study the thionins of the most important grains are compared. After isolation the characteristics of protein structure and the composition of the protein-lipid complex are studied. In addition to chemical analysis the toxicity to yeasts as a biological property is investigated.

1. Materials and methods

1.1. Raw materials

Four cereals were used in the experiments: wheat, rye, barley and oat. For wheat the flour marked BL 112, commercialized for bread manufacture of a maximal ash content of 1.12%, was used. The other three cereals were taken from the market, commercialized for feed purposes.

1.2. Preparation of the thionins and their lipid complexes

1.2.1. Preparation of the sample. The barley, rye and oat were cleaned by physical selection and hand-picking and milled in a mill Type QC 109 (LABOR MIM, Hungary). The flour fractions thus obtained and grits of whole grain as well as the bran were examined. The flours were first extracted in petroleum ether. The parameters of extraction were the same as applied in the extraction of wheat flour (BÉKÉS, 1977).

1.2.2. Preparation of purothionin analogues. The thionins were obtained by the hydrochloric acid method as developed by BALLS and HALE (1940) and modified by BÉKÉS (1977).

For the preparation of lipopurothionin analogues or the stable complex core of thionin and polar lipids the method of REDMAN and FISHER (1969) was used. As it became apparent in an earlier study, a part of phospholipids present in lipopurothionin could not be removed by washing according to FOLCH. Thus to separate protein and lipid components splitting with hydrochloric acid was applied. The course of isolation is illustrated in Fig. 1.

1.3. Analysis of isolates

Two groups of analyses were carried out: chemical and microbiological. In the course of the chemical analyses the nitrogen and phosphorus content of all the samples was determined, to characterize the protein preparations

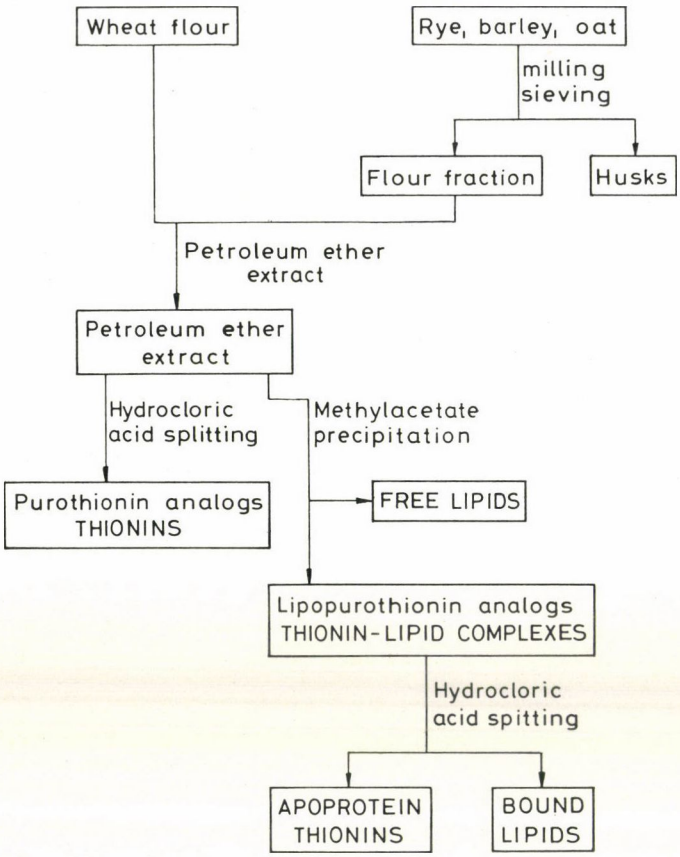

Fig. 1. Isolation of purothionin analogues and lipopurothionin analogues

they were fractionated according to molecular size on *Sephadex* G 75 gel, polyacryl amide gel electrophoresis was applied and the gross amino acid composition of the samples was also established, the lipid fractions, including the free lipid fractions, were subjected to qualitative and quantitative thin-layer chromatography. The methods applied are described in earlier papers (BÉKÉS, 1977; BÉKÉS & SMIED, 1980). Thin-layer chromatography of lipids was complemented by preparing a calibration curve with the standard digalactosyl diglyceride of SERVA (BRD) and determining the digalactosyl diglyceride content.

In the course of microbiological analyses the effect of the isolates (protein, protein fractions, protein–lipid complexes) on *Saccharomyces cerevisiae* and *Saccharomyces uvarum* was studied. Samples containing different amounts of isolates were inoculated with identical cell density and the cell counts were established as a function of time, viable cells were enumerated and morphological changes were followed up by microscopy.

Commercial bakers' yeast was used as *Saccharomyces cerevisiae* culture. The *Saccharomyces uvarum* strain was obtained from the Microbiology Department, UNIVERSITY OF HORTICULTURE, Budapest.

The two media used were prepared (yeast broth glucose, malt extract) as described by HERNANDEZ LUCAS and co-workers (1974) under the same experimental conditions.

2. Results

2.1. Isolation of thionins

Table 1 shows the balance of components in the isolates of the four cereals related to 1 kg flour.

Table 1

Balance of components in flour

Sample	Petroleum ether extract ($g\ kg^{-1}$)	Lipopurothionin analogue ($g\ kg^{-1}$)	Purothionin analogue ($g\ kg^{-1}$)
Wheat flour	11.1	3.03	0.77
Barley flour	16.7	1.77	0.54
Rye flour	12.9	0.75	0.21
Oat grits	55.7	8.83	0.42

It is interesting to note that the proportion of petroleum ether extract to thionins is of the same order of magnitude, in accordance with the protein content of the complex, in wheat, rye and barley, while in the case of oat a similar protein content was found in a four-fold quantity of extract.

2.2. Chemical composition and properties of thionins and their lipid complexes

2.2.1. Analysis of thionins. The elution curves obtained in the fractionation according to molecular size of the proteins, isolated by the method of BALLS, are shown in Fig. 2.

Three main fractions, comparatively easily separated could be obtained from all four cereals. These were mar' ed as in earlier papers as follows: (I+II), III and (IV+V) (BÉKÉS, 1977; BÉKÉS & SMIED, 1980). The percentage distribution of the three main fractions in the four isolates are shown in Table 2.

Fractions (IV+V) of low molecular mass were subjected to more detailed examination. The polyacrylamide gel electropherograms of these fractions are summarized in Fig. 3.

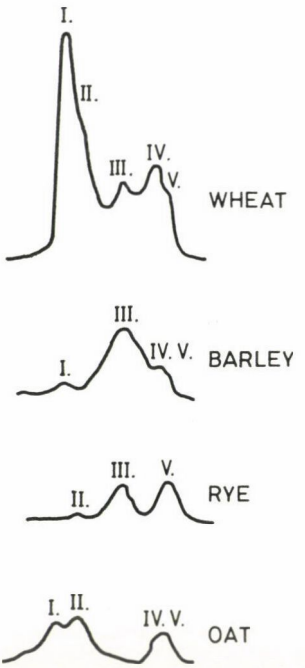

Fig. 2. Elution curves of purothionin analogues on *Sephadex* G 75 columns. a) purothionin (in wheat); b) hordothionin (in barley); c) secalethionin (in rye); d) oat-thionin

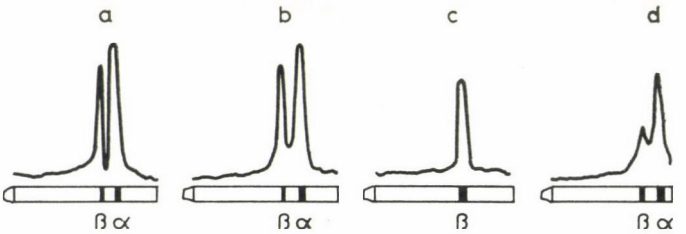

Fig. 3. Electropherograms of the low molecular size fraction (IV + V) of purothionin analogues. a) purothionin (wheat); b) hordothionin (barley); c) secalethionin (rye): d) oat thionin

Table 2

Percentage distribution of thionin fractions on a Sephadex *G 75 column*

Flour	Fractions		
	I + II	III	IV + V
Wheat	57.1	14.6	28.3
Barley	16.7	55.5	27.8
Rye	14.5	41.8	43.7
Oat	68.7	—	31.3

While two bands each may be observed in the electropherograms of wheat, barley and oat, in the rye fraction of low molecular mass only one band is visible. In agreement with data in the literature it may be established that on the basis of its mobility the thionin of rye is pure β fraction.

Table 3

Gross amino acid composition of thionin proteins (%)

Amino acid	Thionins			
	Wheat	Barley	Rye	Oat
ASP	8.92	7.85	8.69	9.57
THR	6.23	7.03	5.66	4.64
SER	7.06	7.85	6.03	6.00
GLU	4.47	3.51	4.49	10.26
PRO	5.11	6.62	6.74	4.05
GLY	8.36	6.95	5.51	6.09
ALA	4.27	5.45	4.49	5.63
CYS	8.05	8.23	7.45	6.42
VAL	5.76	5.45	4.54	5.45
MET	1.58	Trace	0.89	2.40
ILE	4.03	4.14	4.32	3.68
LEU	9.36	10.06	10.52	8.75
TIR	3.84	4.03	4.37	3.73
PHE	4.91	4.48	7.89	4.32
HIS	0.61	0.54	0.50	0.70
LYS	9.82	10.05	9.56	9.46
TRP	—	—	—	—
ARG	8.23	9.85	8.40	8.85

The results of gross amino acid analyses of the isolated proteins are given in Tables 3 and 4.

2.2.2. Analysis of the thionin-lipid complexes. The lipopurothionin analogues obtained by precipitation with methyl acetate from the petroleum ether extracts were split with hydrochloric acid and thus the interrelated lipids and proteins could be separated. In the supernatant of precipitation with methyl acetate the lipids non-interrelated could be investigated.

Table 5 contains the protein–lipid ratio in the complexes and the gross composition of bound lipids while Table 6 contains the gross composition of free lipids.

Table 4

Gross amino acid composition of thionin proteins and their fractions of low molecular mass (%)

Amino acid	Fractions of low molecular mass			
	Wheat	Barley	Rye	Oat
ASP	6.13	6.04	0.91	11.00
THR	4.24	4.76	4.00	8.00
SER	8.35	8.18	7.23	9.02
GLU	2.65	2.81	5.14	0.98
PRO	3.24	3.94	3.84	6.54
GLY	5.02	4.91	2.46	2.34
ALA	3.24	4.01	2.97	3.71
CYS	16.01	15.89	17.51	16.27
VAL	1.34	2.91	2.01	2.74
MET	0.12	—	—	—
ILE	0.73	—	2.00	1.88
LEU	10.24	10.04	9.03	9.00
TIR	2.95	3.01	2.85	3.88
PHE	2.64	2.65	3.14	3.49
HIS	Trace	—	—	—
LYS	14.91	15.41	17.60	14.41
TRP	—	—	—	—
ARG	18.19	15.44	13.31	10.37

Table 5

Protein : lipid ratio and gross lipid composition in thionin-lipid complexes (lipopurothionin analogues)

	Wheat (25.4 : 74.6)	Barley (30.5 : 69.5)	Rye (28.1 : 71.9)	Oat (4.7 : 95.3)
Sterol ester	1.4	1.1	1.6	1.1
Steryl glycoside	1.6	2.4	1.7	1.1
Phosphatidyl ethanol amine	35.7	29.3	33.9	25.4
Phosphatidyl cholin	27.7	24.5	29.7	41.4
Phosphatidyl inositol	6.9	11.5	7.4	7.6
Phosphatidyl serin				
Lysophosphatidyl cholin	—	5.9	4.2	—
Lysophosphatidyl ethanol amine	0.8	3.2	6.5	4.2
Monogalactosyl diglyceride	8.6	5.4	6.5	4.2
Monogalactosyl monoglyceride	0.8	0.2	0.4	0.1
Digalactosyl diglyceride	16.3	16.5	13.3	15.3

Table 6

Gross composition of the free lipids obtained from the supernatant of methyl acetate precipitation in the petroleum ether extract

	Wheat	Barley	Rye	Oat
Sterol ester	5.8	3.4	4.2	5.4
Diglyceride	44.3	41.4	40.9	56.4
1,3 diglyceride	13.7	19.0	14.1	14.5
1,2 diglyceride	4.2	1.6	3.6	2.8
Free sterol	6.2	2.1	4.7	1.1
Free fatty acid	5.6	8.7	10.8	9.8
2-monoglyceride	3.3	1.4	0.8	0.7
Polar lipids	16.9	12.4	10.9	11.2

2.3. Results of microbiological tests

The thionins isolated from all four cereals proved toxic in the course of this study. According to the microscopic study of yeasts grown on media containing thionin, the primary change, appearing already at low toxin content was the formation of floccules. Another consequence of the presence of thionins is the deformation of cells, the appearence of very small cells of 1–3 μm.

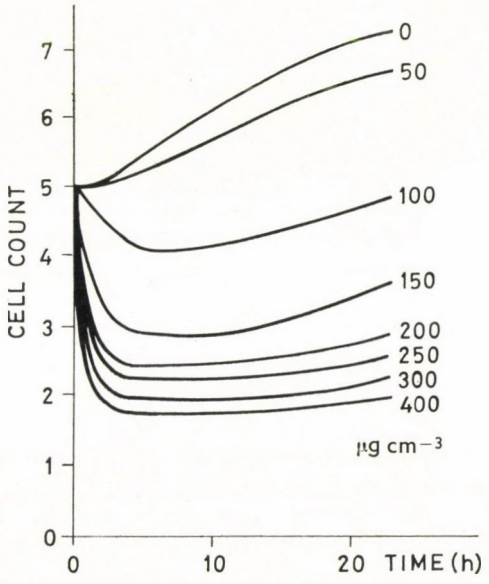

Fig. 4. Growth of *Saccharomyces uvarum* in a medium containing yeast broth and various amounts of purothionin

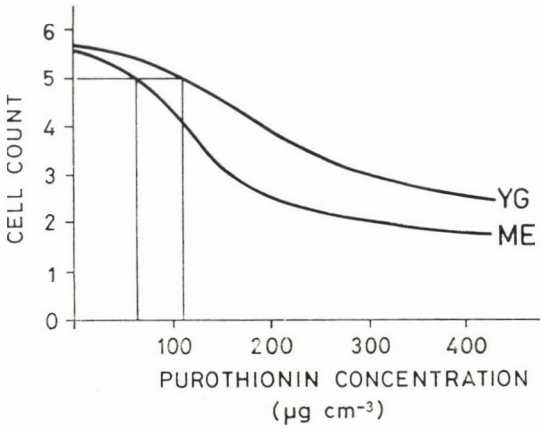

Fig. 5. Parameter introduced to characterize toxicity (thionin level required to maintain cell count at 10^5 cm^{-3} during 5-h cultivation) with *Saccharomyces cerevisiae* in media containing yeast extract (YG) and malt (ME), respectively

Table 7

Comparison of the characteristic toxic levels of various thionins

(Data in the Table show the amount of thionin required to maintain in a 5-h fermentation a cell count of 10^5 cm^{-3})

	Thionin in			
	wheat	barley	rye	oat
	g cm^{-2}			
S. cerevisiae medium:				
yeast extract	120	53	175	185
malt	55	25	60	160
S. uvarum medium:				
yeast extract	35	40	135	175
malt	20	20	82	130

The appearence of toxic symptoms depends on the quantity of the thionin present, with 100 μg of thionin it takes about 15–20 min. The most important consequence of the presence of thionins is the drastic reduction in the viable cell count. In Fig. 4 the viable cell count is shown *vs.* time at different purothionin levels. It may be seen that, in contrast to the pertinent literature, total destruction of cells did not occur even at extremely high toxin levels.

To characterize the toxicity of the isolates or the toxicity at a given level as a function of composition of the medium or yeast strain used, the thionin levels belonging to a cell count of 10^5 cm^{-3} in a 5-h fermentation were accepted. Figure 5 illustrates the determination of this characteristic in the case of purothionin in the fermentation of *S. cerevisiae* in the two media used.

The characteristics thus defined are shown in Table 6 at the parameters tested.

With the lipopurothionin analogues, even at extremely high levels, changes in the cell count proving toxicity were not detected.

3. Conclusions

The final aim of this study was to find out whether protein fractions similar to purothionin obtained from wheat may be isolated from other cereals by the same process used in the case of purothionin and to compare the properties of the proteins thus obtained with those of purothionin.

The results of the experiments have shown the presence of a thionin fraction in the isolates of the three cereals examined: barley, rye and oat. In the case of barley, the findings of the author support those found in the literature. The purothionin analogue in barley was detected about 10 years ago although it has not been thoroughly investigated. As for the thionin fraction isolated from rye, the result is interesting because the authors succeeded in separating secalethionin directly from the petroleum ether extract according to BALLS and HALE (1940, 1944) and not by the indirect method. Report on a thionin analogue in oat was not encountered in the literature, thus this is considered a newly discovered compound. By analogy with names given to thionins detected earlier, the author suggests avenothionin for the isolate from oat.

On studying the cause of the appearence of thionins in the petroleum ether extract, following the findings of HERNANDEZ–LUCAS and co-workers (1977; 1978) as mentioned in the introduction, the digalactosyl diglyceride content of the petroleum ether extracts of flour was determined. No significant difference was found in the digalactosyl diglyceride content of the four extracts. Thus, this compound was not responsible for the inhibition of solubility in the case of rye.

Comparing the data on the quantity of the thionin isolates it was found that independently of the amount of petroleum ether-soluble lipids, the amount of thionins was similar in the four extracts. This is particularly true for the fractions of low molecular mass: in wheat 140, in barley 150, in rye 91 and in oat 131 mg per kg. These data may connect the presence of the thionins to a biological function, hitherto not known but similar in the four plants.

As shown by the results of fractionation according to molecular size all four thionins contain fractions of identical size; only their relative quantities are different according to plant species.

The gel electropherograms of fractions of low molecular mass (IV+V) show them to be analogous in wheat, barley and oat: the two bands characteristic of fractions α and β are seen. The ratio α:β is in all three cases 2:1. In secalethionin, however, in accord with the findings of the Spanish researchers who described it first, only fraction β could be demonstrated.

As for the gross amino acid composition of the samples, a number of analogies may be observed in both the whole preparation and in the fraction of low molecular mass.

In the case of the whole preparations the characteristic thionin properties are striking, *i.e.* the high cystine, lysine and arginine contents, the low glutamic acid and proline levels as well as the triptophan, methionin and hystidin levels unusually low for cereals. However, there are also some striking differences, for instance the high phenylalanin level in rye thionin and the relatively high glutamic acid content of oat.

Similarity is even more striking in the fraction of low molecular mass, though here are also significant differences: *e.g.* complete absence of ILE in the thionin of barley, the very high ASP content in the thionin of oat and the very low GLU level.

In addition to thionins, the thionin-lipid complexes were also analysed. While in an earlier study, the total complex was analysed by the author (BÉKÉS & SMIED, 1980), this time only the complex core (lipopurothionin analogues) precipitated with methyl acetate, containing dominantly polar lipids, was investigated.

It was established from the results that, although the protein : lipid ratio in the four isolated complexes was different, the quality of the lipid fraction as well as the relative proportions of the components are rather similar. Thus it seems that the lipid binding capacity of the four thionin proteins is very similar. The anomaly in the composition of the complex in oat may be explained, accounting for its high lipid content, by the lipid–lipid interaction.

As regards toxicity, the thionins isolated from the four cereals proved to be toxic to about the same degree. Of the two yeast strains, the sensitivity of *S. cerevisiae* is lower than that of *S. uvarum* but it is influenced also by the composition of the medium.

Since toxicity was not observed in the case of thionin–lipid complexes it may be established that, in the toxicity of thionins, the free-binding locations play an important role. In order to prove this, fermentation experiments were carried out with purothionin masked at its lysyl and arginyl groups (BÉKÉS & SMIED, 1981). These chemically modified proteins, just like the protein and lipid complexes, are non-toxic. Indirectly, these experiments

supported the finding of OKADA and YOSHIZUMI (1976) in which they establish that the toxicity in thionin is due to its interrelation with the membrane lipids.

Accordingly, related to the characteristics of purothionin (BÉKÉS & SMIED, 1980), the high lysine and arginine content of thionins as a significant deviation from the general amino acid composition of grain proteins is the unambiguous cause of the two basic characteristics of thionins: the capacity to enter into interaction with lipids and toxicity to microorganisms.

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COMPARATIVE ANALYSIS OF THE PRIMARY STRUCTURE OF GRAIN THIONINS

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The thionins contained in wheat, barley, rye and oat, particularly their fractions molecular mass show a number of analogies in structure and behaviour. These analogies inspired the comparative analysis of the chymotryptic peptides of thionins on the basis of the amino acid sequence of wheat purothionin, the best known member of this group of compounds.

The comparison of the four thionin sequences (α_1-, α_2- and β-purothionin and the viscotoxin) proved the location of 33 out of the 47 amino acids of the low molecular mass fractions to be conservative. The amino acid exchanges in the remaining variable regions may be determined by the comparison of the amino acid composition of their chymotryptic peptides and a probable sequence may be established without arranging in a sequence.

Thionins are biologically active proteins of special chemical composition in cereals. As described in detail in the previous paper (BÉKÉS, 1981), the properties characteristic of the composition, chemical and biological nature of wheat thionin, the best known member of the group, may be demonstrated in the hordothionin of barley, in the secalethionin of rye and in avenothionin, first isolated from oat by the authors. This similarity of the thionins isolated from the various cereals is even more striking in their fractions of low molecular mass, in fractions α and β.

The analogy in their chemical properties and the very close sequential similarity of the purothionin fractions to viscotoxin, the protein isolated from the acorn of oak, suggested the possibility of determining the amino acid sequence in the five thionin fractions isolated by the authors (α- and β-hordothionin, β-secalethionin, α- and β-avenothionin) based on data of their amino acid composition, without arranging them in a sequence.

MAK and JONES (1976), analysing the primary structure of α- and β-purothionin, gave precise and detailed description of the result of their work. This permitted of drawing conclusions as to the sequence of the 17 peptides isolable in the course of chymotrypsin digestion. Assuming the parts of the sequence identical in the three purothionin fractions (α_1, α_2, β) and in the sequence of viscotoxin to be conservative regions, it seemed possible to select the peptides in which the changes are responsible for the character of individual thionins. The comparison of the amino acid pattern of these peptides unambiguously indicates the sequence in different thionin fractions.

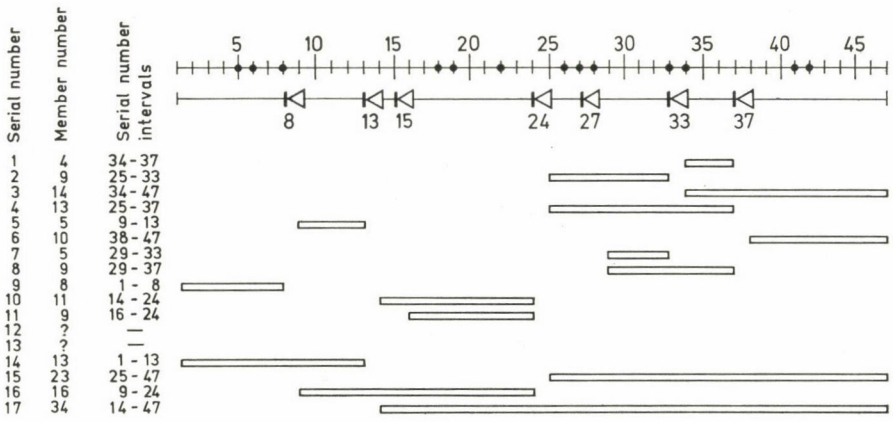

Fig. 1. Location of the chymotryptic peptides of purothionin in the molecule. Detailed information on the Figure is given in the text

The information to be obtained from the data of MAK and JONES (1976) and used in this study is summarized in Fig. 1. In the upper part of the Figure the sequence pattern of thionins may be seen. The parts of the sequence considered to be variable or conservative on the basis of the four thionin fractions of known sequence (α_1-, α_2-, β-purothionin, viscotoxin) are marked. The subsequent section of the Figure illustrates the locations of peptide bonde splitting in the course of chymotrypsin digestion, at the side of the following amino acids: TYR, PHE, LEU, ILE and VAL. On the basis of Tables 2 and 3 in the work of MAK and JONES (1976), the bottom part of the Figure reconstructs the sequence and location in the peptide chain of chymotryptic peptides. The serial numbers corresponding to the succession of elution of peptides (overtaking the serial numbers of α-peptides), the number of peptide members and the location of the peptide in the protein molecule, are shown in Fig. 1.

As can be seen, peptides Nos. 2, 3, 9 and 10 contain all the sequence parts where variable region may be found. Thus, the analysis and comparison if changes and differences in the amino acid pattern of the complete protein can be accounted for, suffice for the comparison of primary structures.

1. Materials and methods

1.1. Isolation of thionins

Purothionin, hordothionin, secalethionin and avenothionin were uniformly produced from the petroleum ether extracts of the pertinent cereals by the hydrochloric cleavage method developed by BALLS and HALE (1940)

for the isolation of purothionin. Isolation was carried out under the conditions as modified by BÉKÉS (1977) and described in detail in papers published earlier (BÉKÉS & SMIED, 1980; BÉKÉS, 1981).

1.2. Production of fractions of low molecular mass

The raw thionin preparations, after cleaning in an appropriate solvent (BÉKÉS, 1977), were fractionated on a *Sephadex* G 75 column according to molecular size (BÉKÉS, 1981).

1.3. Preparation of chymotryptic peptides and their analysis

Further analysis of the thionin fractions was carried out in every detail according to MAK and JONES (1976). Their results were reproduced on the purothionin fractions, then other thionin fractions used in comparison were studied.

Thionin fractions of low molecular mass were separated into fractions α and β on a carboxy methyl cellulose column. Parameters of separation: *Whatman* column of 25×2 cm, type CM 52, linear ammonium acetate gradient (pH = 5.2) in an interval of 0.4–0.7 M.

Using the method of JONES and MAK (1977) an attempt was made to separate subfractions α_1 and α_2, however, this was not successful. Thus, fractions α and β were considered homogeneous preparations. In evaluating the results, however, fraction α was taken into account as a 1:1 mixture of two components.

Fractions α and β were masked first by mercaptoethanol, then by vinyl pyridine. After removal of the masking agents on a *Sephadex* G 25 column chymotrypsin digestion was carried out under the strict observation of the specified parameters.

Peptide fractionation of decisive importance from the point of view of reproduction was carried out on a 25×0.9 cm CM 52 column, applying a pH = 5.2 ammonium acetate gradient elution (in the 0.07–1 M interval).

2. Results

The reproduction of the above mentioned methods, except for the preparation of subfractions α_1 and β_2, was successful. In the case of barley, rye and oat, according to expectations, the fractions obtained were analogous to purothionin. With barley and oat, fractions α and β could be separated and their proportion was, in both cases 2:1. As could be observed in analysis by gel electrophoresis, from secalethionin only one fraction corresponding to β-thionin could be separated (BÉKÉS, 1981).

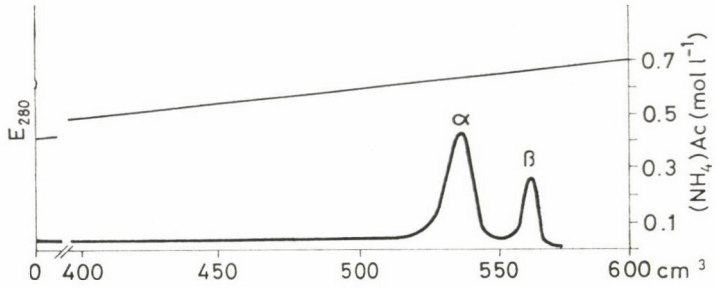

Fig. 2. Fractionation of hordothionin on a CM 52 column

In Fig. 2 the separation on cellulose column of α- and β-fractions from hordothionin is illustrated.

After the chemical modification of the thionin fractions, of the three chymotrypsin digestion methods as given in the reproduced paper (MAK & JONES, 1976), the 30-min digestion proved to be the most satisfactory. The fractions thus obtained were the most reproducible and this digestion permitted to obtain, in one step, the desired four peptide fractions.

Fig. 3 shows the fractionation on a CM-cellulose column of the peptide mixture obtained in the course of chymotrypsin digestion of the α fraction of avenothionin.

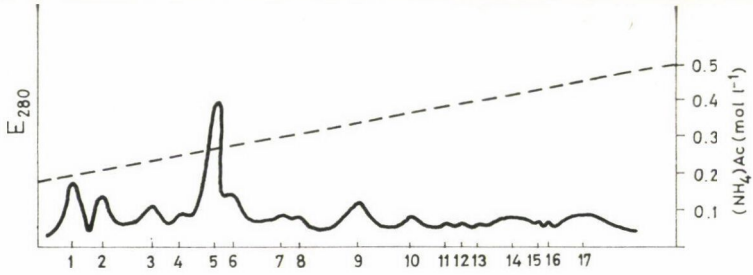

Fig. 3. Elution diagram of the chymotryptic peptides of α avenothionin

The amino acid composition of the thionin fractions or their peptides is given in Tables 1–5. The data contained in the Tables are calculated in mol amino acid per mol protein. In the calculations isoleucin or leucin were considered basic. In addition to the molar data of amino acids the number of amino acid mols obtained by rounding up are also given. Their sum (members of protein or peptide) is to be found in the last row of the Tables. In the case of the α-fractions the number of members, or amino acid mols pertain to two molecules (subfractions α_1 and α_2)thus all the thionins studied have the same number of members (47) or every peptide isolated from the different thionins contains the same number of amino acids.

Table 1

Amino acid composition of the thionin fractions
(mol amino acid per mol protein)

	Barley		Rye	Oat	
	α	β	β	α	β
ASP	4.31 (4)	4.16 (4)	2.91 (3)	7.83 (8)	4.05 (4)
THR	4.94 (5)	1.87 (2)	2.19 (2)	4.13 (4)	4.13 (4)
SER	9.46 (9)	4.69 (5)	4.00 (4)	10.05 (10)	4.42 (4)
GLU	2.45 (2)	1.47 (1)	2.51 (2)	0.37 (0)	0.24 (0)
PRO	4.45 (4)	2.14 (2)	1.58 (2)	5.98 (6)	3.21 (3)
GLY	8.67 (9)	3.06 (3)	4.11 (4)	6.14 (6)	2.16 (2)
ALA	5.03 (5)	2.41 (2)	2.36 (2)	4.21 (4)	2.24 (2)
CYS	16.13 (16)	8.27 (8)	7.64 (8)	15.83 (16)	8.05 (8)
VAL	3.21 (3)	0.91 (1)	0.57 (1)	2.41 (2)	1.06 (1)
ILE	0.21 (0)	0.11 (0)	1.00 (1)	2.00 (2)	0.08 (0)
LEU	9.00 (9)	5.00 (5)	3.52 (5)	7.84 (8)	5.00 (5)
TIR	1.66 (2)	0.98 (1)	0.81 (1)	1.81 (2)	0.83 (1)
PHE	2.20 (2)	1.27 (1)	1.37 (1)	2.14 (2)	0.94 (1)
LYS	11.94 (12)	6.79 (7)	6.54 (7)	11.64 (12)	6.85 (7)
ARS	11.70 (12)	4.83 (5)	4.96 (5)	11.73 (12)	5.06 (5)
Number of members	94	47	47	94	47

Table 2

Amino acid composition in No 2 chymotryptic peptides of thionin fractions
(mol amino acid per mol peptide)

	Wheat		Barley		Rye	Oat	
	α	β	α	β	β	α	β
ASP	0.17 (–)	1.17 (1)	0.21 (–)	1.11 (1)	0.34 (–)	0.30 (–)	0.14 (–)
THR	0.89 (1)	0.36 (–)	1.03 (1)	0.24 (–)	0.21 (–)	2.21 (2)	1.26 (1)
SER	1.14 (1)	0.41 (–)	0.97 (1)	0.14 (–)	0.17 (–)	0.24 (–)	1.06 (1)
GLU	0.31 (–)	0.27 (–)	0.41 (–)	0.31 (–)	0.30 (1)	0.37 (–)	0.34 (–)
PRO	0.42 (–)	0.34 (–)	0.47 (–)	0.21 (–)	0.24 (–)	0.18 (–)	0.21 (–)
GLY	1.17 (1)	0.17 (–)	1.21 (1)	0.17 (–)	0.13 (–)	0.24 (–)	0.40 (–)
ALA	1.08 (1)	1.41 (1)	1.20 (1)	1.37 (1)	0.24 (1)	2.41 (2)	0.34 (–)
CYS	5.98 (6)	2.83 (3)	5.87 (6)	3.21 (3)	3.10 (3)	5.83 (6)	3.06 (3)
VAL	2.04 (2)	1.14 (1)	2.96 (3)	1.17 (1)	0.96 (1)	2.14 (2)	1.14 (1)
ILE	1.00 (1)	0.23 (–)	0.08 (–)	0.21 (–)	1.00 (1)	2.00 (2)	0.08 (1)
LEU	1.36 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.06 (–)	0.41 (–)	1.00 (1)
TIR	0.21 (–)	0.16 (–)	0.21 (–)	0.27 (–)	0.34 (–)	0.24 (–)	0.24 (–)
PHE	0.34 (–)	0.21 (–)	0.41 (–)	0.34 (–)	0.21 (–)	0.37 (–)	0.37 (–)
LYS	2.06 (1)	1.06 (1)	2.41 (2)	2.16 (2)	1.17 (1)	2.34 (2)	1.14 (1)
ARG	1.89 (2)	0.94 (1)	1.96 (2)	1.89 (2)	1.23 (1)	1.97 (2)	0.96 (1)
Number of members	18	9	18	9	9	18	9

Table 3

Amino acid composition in No 3 chymotryptic peptides of thionin fractions
(mol amino acid per mol peptide)

	Wheat		Barley		Rye	Oat	
	α	β	α	β	β	α	β
ASP	0.27 (–)	0.98 (1)	0.34 (–)	1.11 (1)	1.08 (1)	1.84 (2)	1.31 (1)
THR	1.13 (1)	1.35 (1)	1.14 (1)	1.45 (1)	1.27 (1)	0.36 (–)	1.14 (1)
SER	5.21 (5)	2.34 (2)	5.14 (5)	2.08 (2)	1.98 (2)	6.21 (6)	2.08 (2)
GLU	0.37 (–)	0.45 (–)	0.21 (–)	0.34 (–)	0.34 (–)	0.41 (–)	0.39 (–)
PRO	3.69 (4)	1.87 (2)	3.87 (4)	1.98 (2)	1.85 (2)	4.29 (4)	2.14 (2)
GLY	4.14 (4)	1.08 (1)	4.06 (4)	1.13 (1)	1.07 (1)	2.43 (2)	1.34 (1)
ALA	0.35 (–)	0.41 (–)	0.37 (–)	0.24 (–)	0.37 (–)	0.17 (–)	0.48 (–)
CYS	1.87 (2)	1.06 (1)	1.96 (2)	1.13 (1)	1.19 (1)	1.87 (2)	1.14 (1)
VAL	0.24 (–)	0.13 (–)	0.31 (–)	0.47 (–)	0.34 (–)	0.31 (–)	0.47 (–)
ILE	0.24 (–)	0.17 (–)	0.27 (–)	0.34 (–)	0.41 (–)	0.14 (–)	0.29 (–)
LEU	2.00 (2)	1.00 (1)	2.00 (2)	1.00 (1)	1.00 (1)	2.00 (2)	1.00 (1)
TIR	0.34 (–)	0.29 (–)	0.41 (–)	0.34 (–)	0.47 (–)	0.39 (–)	0.34 (–)
PHE	2.41 (2)	1.34 (1)	2.29 (2)	1.14 (1)	0.89 (1)	1.88 (2)	1.17 (1)
LYS	5.74 (6)	3.27 (3)	5.61 (6)	3.29 (3)	3.24 (3)	5.96 (6)	3.14 (3)
ARG	1.98 (2)	1.14 (1)	1.97 (2)	1.14 (1)	1.21 (1)	1.97 (2)	1.08 (1)
Number of members	28	14	28	14	14	28	14

Table 4

Amino acid composition in No 9 chymotryptic peptides of thionin fractions
(mol amino acid per mol peptide)

	Wheat		Barley		Rye	Oat	
	α	β	α	β	β	α	β
ASP	0.31 (–)	0.24 (–)	01.9 (–)	0.24 (–)	0.18 (–)	1.87 (2)	1.11 (1)
THR	2.94 (3)	1.16 (1)	2.84 (3)	1.16 (1)	1.21 (1)	2.08 (2)	1.34 (1)
SER	3.15 (3)	2.34 (2)	3.06 (3)	2.24 (2)	2.37 (2)	2.14 (2)	1.06 (1)
GLU	0.41 (–)	0.36 (–)	0.31 (–)	0.21 (–)	0.31 (–)	0.34 (–)	0.27 (–)
PRO	0.20 (–)	0.41 (–)	0.24 (–)	0.31 (–)	0.19 (–)	0.41 (–)	0.34 (–)
GLY	0.34 (–)	0.39 (–)	0.31 (–)	0.34 (–)	0.37 (–)	0.39 (–)	0.41 (–)
ALA	0.24 (–)	0.17 (–)	0.18 (–)	0.11 (–)	0.24 (–)	0.17 (–)	0.28 (–)
CYS	3.89 (4)	2.34 (2)	3.71 (4)	2.06 (2)	2.43 (2)	4.36 (4)	2.17 (2)
VAL	0.34 (–)	0.40 (–)	0.34 (–)	0.31 (–)	0.41 (–)	0.21 (–)	0.37 (–)
ILE	0.17 (–)	0.24 (–)	0.19 (–)	0.27 (–)	0.35 (–)	0.37 (–)	0.39(–)
LEU	2.00 (2)	1.00 (1)	2.00 (2)	1.00 (1)	1.00 (1)	2.00 (2)	1.00 (1)
TIR	0.37 (–)	0.24 (–)	0.31 (–)	0.08 (–)	0.24 (–)	0.34 (–)	0.39 (–)
PHE	0.41 (–)	0.31 (–)	0.38 (–)	0.11 (–)	0.39 (–)	0.41 (–)	0.47 (–)
LYS	2.13 (2)	2.06 (2)	2.18 (2)	2.34 (2)	1.17 (1)	2.38 (2)	1.08 (1)
ARG	1.86 (2)	0.17 (–)	1.89 (2)	0.06 (–)	0.89 (1)	2.08 (2)	0.16 (–)
Number of members	16	8	16	8	8	16	8

Table 5

Amino acid composition in No 10 chymotryptic peptides of thionin fractions
(mol amino acid per mol peptide)

	Wheat		Barley		Rye	Oat	
	α	β	α	β	β	α	β
ASP	1.98 (2)	1.41 (1)	1.84 (2)	1.24 (1)	1.17 (1)	2.06 (2)	1.43 (1)
THR	0.14 (–)	0.27 (–)	0.17 (–)	0.29 (–)	0.09 (–)	0.21 (–)	1.00 (1)
SER	1.00 (1)	0.25 (–)	0.41 (–)	1.00 (1)	1.00 (1)	2.00 (2)	0.21 (–)
GLU	2.35 (2)	1.14 (1)	2.37 (2)	1.41 (1)	0.47 (–)	2.24 (2)	0.47 (–)
PRO	0.17 (–)	0.37 (–)	0.28 (–)	0.34 (–)	0.21 (–)	0.34 (–)	1.34 (1)
GLY	2.14 (2)	1.00 (1)	2.21 (2)	1.47 (1)	2.37 (2)	2.41 (2)	0.27 (–)
ALA	3.05 (3)	2.41 (2)	4.49 (4)	0.65 (1)	1.19 (1)	1.88 (2)	2.14 (2)
CYS	1.96 (2)	0.90 (1)	2.01 (2)	0.84 (1)	0.67 (1)	1.79 (2)	0.87 (1)
VAL	0.24 (–)	0.34 (–)	0.24 (–)	0.17 (–)	0.08 (–)	0.14 (–)	0.34 (–)
ILE	0.17 (–)	0.08 (–)	0.00 (–)	0.21 (–)	0.21 (–)	0.31 (–)	0.24 (–)
LEU	4.41 (4)	2.47 (2)	4.45 (4)	1.74 (2)	2.14 (2)	2.21 (2)	2.13 (2)
TIR	0.32 (–)	0.24 (–)	0.17 (–)	0.21 (–)	0.27 (–)	0.38 (–)	0.41 (–)
PHE	0.21 (–)	0.35 (–)	0.08 (–)	0.37 (–)	0.21 (–)	0.24 (–)	0.34 (–)
LYS	2.35 (2)	1.14 (1)	2.00 (2)	0.97 (1)	1.08 (1)	2.21 (2)	1.11 (1)
ARG	4.18 (4)	2.36 (2)	4.14 (4)	2.41 (2)	2.36 (2)	4.06 (4)	2.06 (2)
Number of members	22	11	22	11	11	22	11

3. Conclusions

3.1. Comparative evaluation of the primary structure of the thionin fractions

Using the processed data of Table 1, the deviation in the amino acid composition of the two α- and the three β-fractions from that of the α- and β-purothionin fractions of known sequence is summarized in Table 6. The Table contains the molecular amino acid composition of α-purothionin (*i.e.* $\alpha_1 + \alpha_2$) and of β-purothionin and the deviations as measured in the other thionins.

From the data the following conclusions may be drawn:

– Every thionin fraction consists of the same number (47) of amino acids.

– There is little difference in the amino acid composition of the fractions: the difference is smallest in hordothionin (exchange of only one amino acid) while the greatest deviation was found in α-avenothionin, where 7 amino acids differ from the corresponding wheat fractions. However, the exchange of 7 amino acids pertains to two molecules (α_1 and α_2).

– Considering the quality of the unchanged amino acids, no difference is observed in the number of the amino acids characteristic of the thionins. Thus 8 cystein molecules are in each thionin molecule and the number of basic amino acids is also the same.

Table 6

Comparison of the amino acids in thionin fractions with the α and β fractions of purothionin

	Wheat α	Barley α	Oat α	Wheat β	Barley β	Rye β	Oat β
ASP	4	=	+4	4	=	−1	=
THR	5	=	−1	2	=	=	+2
SER	10	−1	=	4	+1	=	=
GLU	2	=	−2	1	=	+1	−1
PRO	4	=	+2	2	=	=	+1
GLY	9	=	−3	3	=	+1	−1
ALA	4	+1	=	3	−1	−1	−1
CYS	16	=	=	8	=	=	=
VAL	2	+1	=	1	=	=	=
ILE	1	−1	+1	=	=	+1	=
LEU	9	=	−1	5	=	−1	=
TIR	2	=	=	1	=	=	=
PHE	2	=	=	1	=	=	=
LYS	12	=	=	7	=	=	=
ARG	12	=	=	5	=	=	=

– In the mixture of peptides obtained by chymotrypsin digestion, aromatic amino acids were found only in the conservative parts of sequence and the number of amino acids with hydrophobic side chain is also constant (ILE + LEU + VAL). Thus, in everyone of the thionin fractions the number of peptide bonds hydrolysed by chymotrypsin is the same.

Tables 7–10 are of the same construction as Table 6: the amino acid data of the purothionin peptides of known sequence are compared to those of barley, rye and oat.

Accepting the assumption that the parts of the sequence found identical in the three known purothionins and in the viscotoxin of oak are considered conservative, and that, thus, these may be found in the cereal thionins, too, the sequential locations of changes may be established. As regards the amino acids of changed location, the following conclusions may be drawn, using the data found in Tables 7–10:

Table 7

Comparison of the amino acid composition in No 2 α and β peptides

	Wheat α	Barley α	Oat α	Wheat β	Barley β	Rye β	Oat β
ASP	—	=	=	1	=	−1	−1
THR	1	=	+1	—	=	=	+1
SER	1	=	−1	—	=	=	+1
GLU	—	=	=	—	=	+1	=
PRO	—	=	=	—	=	=	=
GLY	1	=	−1	—	=	=	=
ALA	1	=	+1	1	=	=	−1
CYS	6	=	=	3	=	=	=
VAL	2	+1	=	1	=	=	=
ILE	1	−1	+1	—	=	+1	=
LEU	1	=	−1	1	=	−1	=
TIR	—	=	=	—	=	=	=
PHE	—	=	=	—	=	=	=
LYS	2	=	=	1	=	=	=
ARG	2	=	=	1	=	=	=
Number of exchanges		1	3		0	2	2

Table 8

Comparison of the amino acid composition in No 3 α and β peptides

	Wheat α	Barley α	Oat α	Wheat β	Barley β	Rye β	Oat β
ASP	—	=	+2	1	=	=	=
THR	1	=	−1	1	=	=	=
SER	5	=	+1	2	=	=	=
GLU	—	=	=	—	=	=	=
PRO	4	=	=	2	=	=	=
GLY	4	=	−2	1	=	=	=
CYS	2	=	=	1	=	=	=
VAL	—	=	=	—	=	=	=
ILE	—	=	=	—	=	=	=
LEU	2	=	=	1	=	=	=
TIR	—	=	=	—	=	=	=
PHE	2	=	=	1	=	=	=
LYS	6	=	=	3	=	=	=
ARG	2	=	=	1	=	=	=
Number of exchanges		0	3		0	0	0

Table 9

Comparison of the amino acid composition in No 9 α and β peptides

	Wheat α	Barley α	Oat α	Wheat β	Barley β	Rye β	Oat β
ASP	—	=	+2	—	=	=	+1
THR	3	=	−1	1	=	=	=
SER	3	=	−1	2	=	=	−1
GLU	—	=	=	—	=	=	=
PRO	—	=	=	—	=	=	=
GLY	—	=	=	—	=	=	=
ALA	—	=	=	—	=	=	=
CYS	4	=	=	2	=	=	=
VAL	—	=	=	—	=	=	=
ILE	—	=	=	—	=	=	=
LEU	2	=	=	—	=	=	=
TIR	—	=	=	—	=	=	=
PHE	—	=	=	—	=	=	=
LYS	2	=	=	2	=	=	=
ARG	2	=	=	—	=	=	=
Number of exchanges		0	2		0	0	1

Table 10

Comparison of the amino acid composition in No 10 α and β peptides

	Wheat α	Barley α	Oat α	Wheat β	Barley β	Rye β	Oat β
ASP	2	=	=	1	=	=	=
THR	—	=	=	—	=	=	=
SER	1	−1	+1	—	+1	=	=
GLU	2	=	−2	1	=	=	−1
PRO	—	=	+2	—	=	=	+1
GLY	2	=	=	1	=	+1	=
ALA	3	+1	−1	2	−1	−1	=
CYS	2	=	=	1	=	=	=
VAL	—	=	=	—	=	=	=
ILE	—	=	=	—	=	=	=
LEU	4	=	=	2	=	=	=
TIR	—	=	=	—	=	=	=
PHE	—	=	=	—	=	=	=
LYS	2	=	=	1	=	=	=
ARG	4	=	=	2	=	=	=
Number of exchanges		1	3		1	1	1

– The amino acid in the changed location may be unambiguously established if there is only one change within one peptide. This is the case with α-hordothionin and in the 10th peptide of the three β-thionins.

– The exchange is similarly unambiguous if two amino acids are exchanged for the same amino acid. This is the case with the 9th peptide of α-avenothionin.

– Considering the selective cleaving capacity of chymotrypsin, without change in the peptide distribution, LEU, ILE, VAL, PHE, TYR may be exchanged only among themselves.

On the basis of the above the exchanges as marked in Tables 7–10 may be localized to the appropriate place in the sequence.

The correspondence of the variable amino acids is of a measure, based on data in the Tables, enabling the establishment of the sequences of subfractions α_1 and α_2, too. Here the only assumption applied was that, where possible, the variable amino acids were located in accord with the known sequence in α_1- and α_2-purothionins. In the case of the 2nd peptide, for instance in fraction α of hordothionin, in the amino acid locations 26 and 27 α_1 = ALA–GLY and α_2 = SER–THR variants were applied because, these amino acids are in the corresponding locations of wheat purothionin. Theoretically, variants α_1 = SER–THR and α_2 = ALA–GLY are also possible, even variants α_1 = SER–GLY and α_2 = ALA–THR could not be excluded.

Data for avenothionin show that the α fraction does not contain subfractions α_1 and α_2. The number and character of the variable amino acids excludes this possibility.

Comparing the data for proteins in Table 6 with the data for peptides in Tables 7, 8, 9, 10 it may be established that the number of exchanges in peptides agrees with the number of exchanges observed in proteins. In addition to the four thionins described in the literature the six samples analysed in the present study produced exchanges of amino acids of the same serial number and the conservative regions are found in all ten cases.

On the basis of the above the thionin sequences are characterized in Table 11. As can be seen in this Table, out of the 47 amino acids, the location of 33 is conservatively fixed and variation is possible in 14 locations.

Certain regularity, however, may be discovered in the amino acid exchanges, too:

– Basic amino acids are exchanged only by basic amino acids (exceptions are: the 5th location, where a basic place is taken by PRO and in the 19th place of viscotoxin where THR takes the place of ARG).

– Leucin, isoleucin and valin are exchanged only among themselves.

– ALA, THR, SER, GLY and in some cases ASP appear equivalent in changeable locations. In certain locations, however, only given exchanges are permissible (*e.g.* in the 26th ALA or SER).

Table 11

Amino acid sequence in thionins

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

NH_2–LYS–SER–CYS–CYS–X_1–X_2–THR–X_3–GLY–ARG–ASP–X_4–TIR–ASP–LEU–CYS–ARG–X_5–X_6–GLY–ALA–X_7–LYS–LEU–

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47

CYS–X_8–X_9–X_{10}–CYS–ARG–CYS–LYS–X_{11}–X_{12}–SER–GLY–LEU–SER–CYS–PRO–X_{13}–X_{14}–PHE-PRO-LYS-ARG-LYS-COOH

	Serial number	Purothionin			Hordothionin			Secale-thionin	Avenothionin		Viscotoxin
		α_1	α_2	β	α_1	α_2	β		α	β	
X_1	5	ARG	ARG	LYS	ARG	ARG	LYS	LYS	ARG	LYS	PRO
X_2	6	SER	THR	SER	SER	THR	SER	SER	ASP	ASP	ASP
X_3	8	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	THR
X_4	12	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	ILE
X_5	18	ALA	SER	ALA	ALA	ALA	SER	GLY	SER	ALA	LEU
X_6	19	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	THR
X_7	22	GLU	GLU	GLU	GLU	GLU	GLU	GLU	PRO	PRO	PRO–THR
X_8	26	ALA	SER	ALA	ALA	SER	ALA	ALA	ALA	SER	ALA
X_9	27	GLY	THR	ASP	GLY	THR	ASP	GLU	THR	THR	LYS
X_{10}	28	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	VAL
X_{11}	33	ILE	LEU	LEU	VAL	LEU	LEU	ILE	ILE	LEU	LEU
X_{12}	34	SER	THR	THR	SER	THR	THR	THR	SER	THR	ILE
X_{13}	41	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	–
X_{14}	42	GLY	GLY	ASP	GLY	GLY	ASP	ASP	ASP	ASP	SER

– Sequential relations are much more close in wheat, barley and rye than in viscotoxin, where exchanges are more frequent, while the oat thionin takes a transitory position. This close relation is shown *e.g.* in the 6th location where in the case of purothionin, hordothionin and secalethionin SER may be exchanged only for THR, while in the case of avenothionin and viscotoxin for ASP.

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FACTORS AFFECTING PECTINESTERASE ACTIVITY

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In the present study the pectinesterase (PE) component of a pectin lyase preparation, obtained by alcoholic precipitation from the filtrate of an *Aspergillus niger* culture, was investigated. The optimum parameters and those affecting enzyme activity in the negative direction, were established.

The temperature optimum of the above PE component was found to be at 30 °C while its optimal pH was 4.5.

In the enzyme concentration range between 0.06 and 0.6 mg cm^{-3}, PE activity was linearly related to concentration during 15- and 30-min reaction periods.

The reaction rate of the substrate (*Pomosin* pectin esterified to 70%) increased from 0.262 to 0.438 mval min^{-1} with the enzyme concentration increasing from 0.15 to 1.0%.

The respective values of V_{max} and K_m were found to be 0.477 min^{-1} and 1.445 mg cm^{-3}.

In the course of its action, pectinesterase hydrolyses the methyl ester groups of pectin while galacturonic acid and methyl alcohol accumulate in the reaction medium (Fig. 1).

Pectinesterase was isolated from a great number of plants and vegetables (SOLMS & DEUEL, 1955; NAGEL & PATTERSON, 1967; LEE & MACMILLAN, 1968; POZSÁR-HAJNAL & POLACSEK–RÁCZ, 1975). It may be found also in the pectin complexes produced by different microorganisms (PHAFF, 1947; COLE & WOOD, 1961; ENDO, 1964; REXOVA-BENKOVA & SLEZARIK, 1966).

Its presence is extremely important in enzyme complexes which do not contain pectin lyase but other depolymerising enzyme components as endo-polygalacturonase or pectate or pectic acid lyase. The latter are capable of breaking down the pectin molecule only if it had been previously affected by pectinesterase.

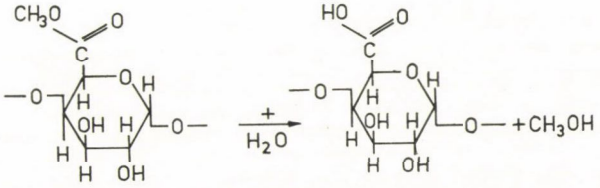

Fig. 1. Mechanism of action by pectinesterase

A disadvantageous consequence of the effect of PE is, that in the course of the reaction, methyl alcohol, harmful to human health, accumulates in the product. Therefore, optimization should aim at maximal pectin decomposition with minimal methanol accumulation (DAHODWALA *et al.*, 1974).

The presence of PE in fruit juice concentrates is not desirable because, by saponifying the pectins, the enzyme produces low-ester pectins which coagulate with Ca^{2+} in the fruit, forming Ca-pectate. Fruit juices produced from such concentrates are not adequately stable (ROMBOUTS & PILNIK, 1971).

The presence in larger quantities of PE in clarifying enzymes is furthermore not desirable (JAKOB *et al.*, 1973) because it affects the volatile ester content of the juices, thereby causing changes of aroma, which are mostly disadvantageous and are more easily detected by odour changes than by changes in taste.

1. Materials and methods

1.1. The enzyme preparation used

The enzyme preparation was obtained from the culture filtrate of an *Aspergillus niger* strain (a variant isolated after Na-azide treatment, ZETELAKI-HORVÁTH *et al.*, 1979) by precipitation with ethylalcohol. PE activity of the preparation was: 177 mval $min^{-1} g^{-1}$.

1.2. Measurement of the enzyme activity

The substrate used for enzyme activity measurement was a 1% solution of *Pomosin* pectin (POMOSIN GmbH, FRG) in acetate buffer of pH 4.5 and 5 cm^3 enzyme solution. The reaction mixture was incubated for 15 min at 30 °C in a water bath. The hydrolyzing effect of the enzyme was determined by titration with a 0.1 *N* NaOH solution. The activity measurement was carried out on a *"Pulse" Titrimeter Automatic* (Type OP 504, FŐVÁROSI FINOMMECHANIKAI VÁLLALAT, Budapest). Activity was calculated as follows:

$$\mathrm{PE} = \frac{a \cdot F \cdot 100}{t \cdot s}$$

where

a = difference between the titration values of the control and the sample, in cm^3 of 0.1 *N* NaOH

F = factor of the NaOH solution

100 = milliequivalent of hydrolyzed ester bonds corresponding to 1 cm^3 0,1 *N* NaOH solution

t = duration of hydrolysis (min)
s = amount of enzyme used in the activity measurement (g or cm^3 fermentation broth)

The activity of the enzyme was characterized by the number of milli-equivalent ester bonds hydrolysed per minute.

1.3. Mathematical statistical evaluation

From the three parallel measurements deviation was calculated and the significance level of the results was established by the t test.

To establish the kinetic constants, the *Lineweaver–Burk* method of regression calculation was used.

2. Results

2.1. The effect of temperature

The effect of temperature was measured with a 1% *Pomosin* substrate in a reaction mixture containing 0.66 mg cm^{-3} enzyme preparation in the temperature range between 20 and 60 °C at 10 °C intervals.

The best effect was obtained at 30 °C (Fig. 2).

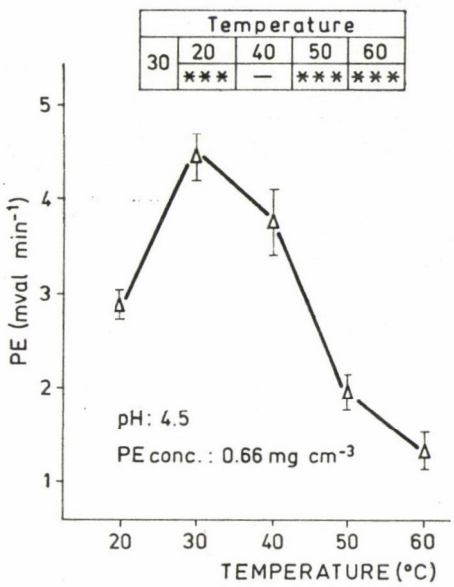

Fig. 2. The effect of temperature on pectinesterase activity. Comparison by the t test of activities measured at 30 °C with those measured at other temperatures
— = non-significant ($P \geq 95\%$)
*** = very highly significant ($P \leq 99.9\%$)

There was no significant difference in the activity at 30 or 40 °C, but the activity measured at 30 °C was significantly higher than those measured at 20, 50 or 60 °C.

2.2. The effect of pH

The effect of pH was studied at 30 °C with a substrate containing 1% *Pomosin* in a reaction mixture containing 0.66 mg cm^{-3} enzyme preparation.

Measurements were carried out at the following pH values: 3.0, 4.0, 4.5, 5.0, 5.5 and 6.0 (Fig. 3).

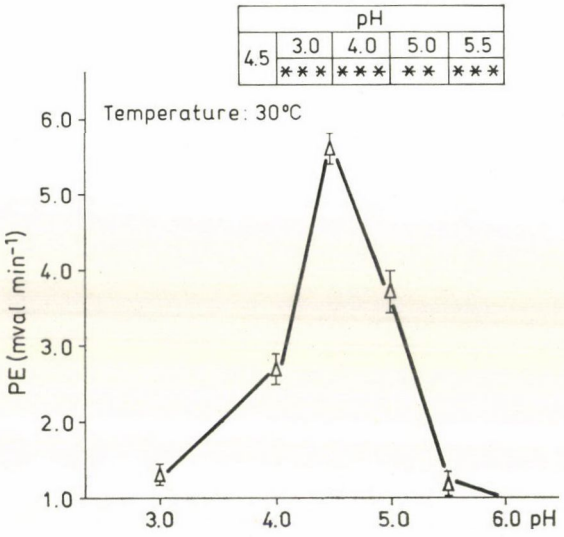

Fig. 3. Pectinesterase activity as affected by pH. Comparison of enzyme activity as measured at pH 4.5 with those measured at other pH values.
** = highly significant ($P \leq 99\%$)
*** = very highly significant ($P \leq 99.9\%$)

Out of the different pH values applied, pH 4.5 affected most advantageously the activity of the enzyme. This was highly or very highly significantly higher than those measured at all other pH values tested.

2.3. The effect of enzyme concentration

The effect of enzyme concentration was investigated at a substrate concentration of 1% and pH 4.5 using incubation periods of 15, 30, 60, 90 and 120 min, resp. (Fig. 4).

With increasing enzyme concentration, the reaction rate increased linearly up to a certain concentration. With reaction periods of 15 and 30 min,

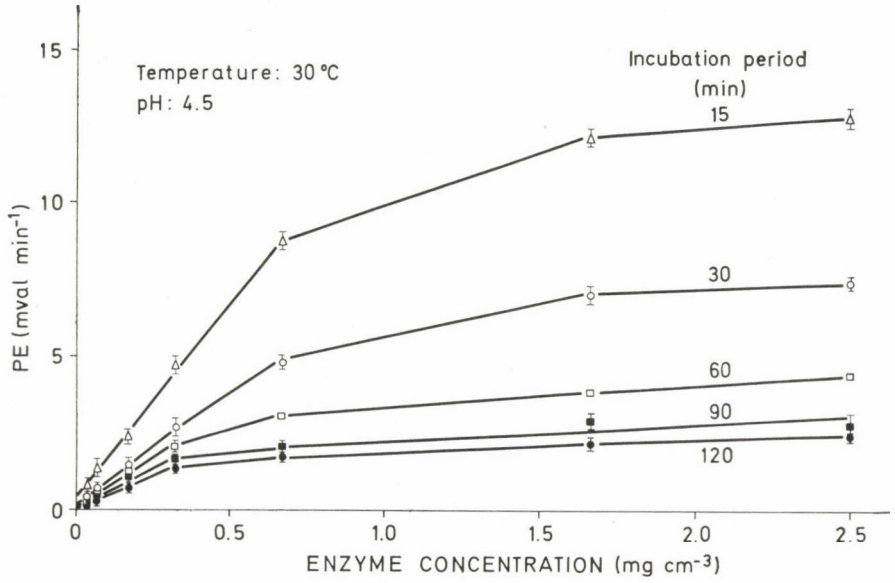

Fig. 4. Pectinesterase activity as affected by enzyme concentration and incubation period (substrate concentration: 10 mg cm^{-3})

the linear section ended at the enzyme preparation concentration of 0.66 mg cm^{-3}, while with reaction periods of 60, 90 and 120 min, the same happened at an enzyme concentration of 0.33 mg cm^{-3}.

2.4. The effect of substrate concentration

The effect of substrate concentration was studied in a reaction mixture of 0.33 mg cm^{-3} enzyme preparation and pH 4.5, at 30 °C, using incubation periods of 15, 30, 45, and 60 min, respectively (Fig. 5).

No change was observed in the reaction rate when the substrate concentration was increased from 1.5 mg cm^{-3} to 2 mg cm^{-3}. By increasing it from 2.0 mg cm^{-3} to 4, 7.5 or 10 mg cm^{-3}, however, the rate increased 1.19- 1.75- and 1.89-fold, respectively.

2.5. Kinetic constants of the enzyme

The reciprocals of the reaction rates as measured at different substrate concentrations were plotted against the reciprocals of the substrate concentration and the slope of the line and its point of intersection with the ordinate were determined by regression calculation, then the K_m value was determined and found to be 1.445 mg cm^{-3} and the maximum reaction rate 0.474 mval min^{-1}.

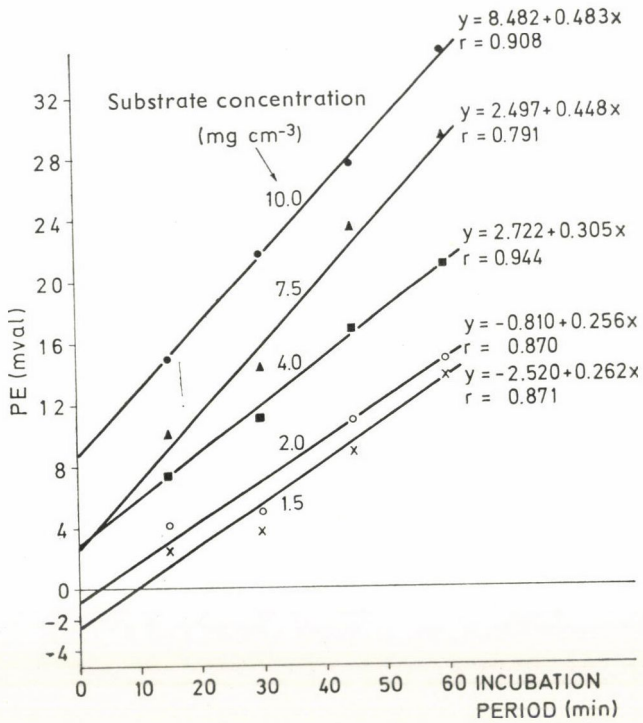

Fig. 5. Pectinesterase activity at different substrate concentrations as affected by the incubation period

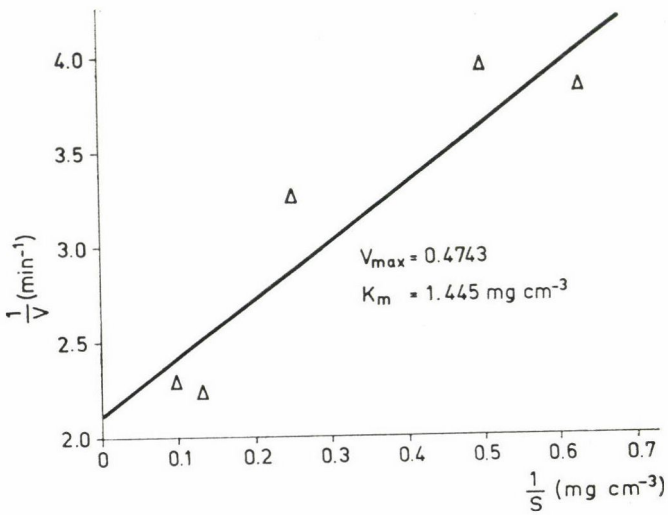

Fig. 6. Graphical representation according to *Lineweaver-Burk* of the relation between PE activity and substrate concentration

3. Conclusions

On the basis of kinetic measurements it was established that instead of the reaction period of 60 min as hitherto used for measurement in the linear section of reaction rate, a 15 min period is more suitable.

The concentration range of the enzyme preparation suitable for measurement was 0.06–0.6 mg cm^{-3} and the optimum of substrate concentration was 10 mg cm^{-3}.

The optimal temperature of PE activity was 30 °C and the most suitable pH was 4.5 for the enzyme preparation studied. This is slightly lower than that found by CALESNIK and co-workers (1950) and OI and SATOMURA (1965) for PE of fungal origin (4.8–5.1).

MCCOLLOCH and KERTESZ (1948) found the fungal enzyme rapidly inactivating at 40 °C while the enzyme originating from tomatoes remained stable during 1 h at 60 °C. On the other hand the enzyme of tomato origin is inactivated at pH 3.5 while the fungal preparation remains active at this pH.

The K_m value of 1.445 mg cm^{-3} as found in the present study for the PE component of lyase type enzyme complexes fairly approximates the value established by DAHODWALA and co-workers (1974) for PE preparations of *Aspergillus niger* (2 mg cm^{-3}).

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DYNAMICS OF CAP OPENING IN *AGARICUS BISPORUS* AND CHANGES IN SPORE NUMBERS *VS.* CAP OPENING AND RADIATION TREATMENT

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The spore count in *Agaricus bisporus*, treated with radiation doses of 1.5 and 2.5 kGy was studied as a function of cap opening and radiation treatment (at 14–16 °C and RH 80–90%).

It was established that spore formation starts in the mushroom while the cap is still closed and opening of the pileus starts when the spore number begins to increase.

In treated mushrooms not only the cap remains closed but also the development of gills and thereby the development of spores is inhibited. Maturation retardation becomes apparent in the inhibition of the increase in numbers and of the darkening of the spores.

STADEN (1964; 1965a, b, c, d; 1966) was the first to report on a new method for the extension of the storage life of mushrooms. Experiments have shown irradiation to inhibit the opening of pileus and changes in the sensory properties (STOLLER, 1968; GILL, 1968; GILL *et al.*, 1969; KOVÁCS *et al.*, 1968 a, b; KOVÁCS & VAS, 1970; KOVÁCS & VAS, 1974a, b; AOKI *et al.*, 1974; SKOU *et al.*, 1974; LANGERAK, 1976).

So far researchers have not investigated the spores during storage subsequent to radiation treatment.

A mass of *Agaricus bisporus* spores is of purple-brownish or blackish-brownish colour and 4–8 × 3–6 μm in size. When the gills are developed, the development of the spores begins. In cultivated mushrooms there are two spores to each basidium and two nuclei in each spore and the subsequent division retains the number. Thus, a mature spore contains four haploid nuclei (BOHUS *et al.*, 1961). Sometimes it occurs that there are three or one spore on the basidium of cultivated mushrooms. This is an anomaly and the frequency of such basidia depends on the maturity of individual fruiting bodies and the number of flushes (PELHAM, 1965). Lately it has been shown that the spores in *Agaricus bisporus* do not mature all at the same time, basidia containing mature spores may be found in unripe fruiting bodies, too (ELLIOT, 1977).

The cell wall of the spores of *Agaricus bisporus* consists of three layers. The outer layer is of 300, the middle layer of 150 μm thickness. The inner layer is the thinnest (20 μm). The outer layer is of uniform thickness and dyes well, the middle layer is of looser structure and the inner one is pellicular. Within

the three layered cell wall is the plasma membrane, a vacuole-like particle, the nucleus, the ribosome, the mitochondrion, a microbody, lipid granules and glycogen (GREUTER & RAST, 1975).

The aim of this study was to establish the change in the spore count in radiation-treated mushrooms during subsequent storage.

1. Materials and methods

1.1. Raw material

Agaricus bisporus was obtained from the DUNA AGRICULTURAL CO-OPERATIVE. Mushrooms of 3–5 cm pileus diameter were used in the experiments because in earlier studies these were found to form a homogeneous population (KOVÁCS *et al.*, 1968).

1.2. Radiation treatment

Radiation treatment was carried out with the panoramic radiation source of appr. 3.07 PBq ^{60}Co activity of the INSTITUTE OF ISOTOPES OF THE HUNGARIAN ACADEMY OF SCIENCES.

Doses applied were: 0, 1.5 and 2.5 kGy

Dose rate: 2.5 kGy · h^{-1}

1.3. Storage

The mushrooms were stored at 14–16 °C in a cooling-heating thermostat at 80–90% RH (relative humidity).

1.4. Determination of the opening of the cap

The degree of opening of the cap is understood to mean the opening expressed in percentage of initial cap diameter. On the basis of preliminary experiments degrees of opening were grouped as follows:

0.	closed cap	0
1.	degree of opening	1–10%
2.	,, ,,	11–20%
3.	,, ,,	21–28%
4.	,, ,,	29–36%
5.	,, ,,	37– %

Since the opening was measured only on one side of the cap, at the full degree of opening it would amount to 50%. However, in reality this value is lower because of the presence of the stipe.

Earlier studies have shown that the dynamics of opening is not linear with time. The following experiment was intended to prove the above.

The RESEARCH FILM STUDIO OF THE INSTRUMENT AND MEASURING TECHNICAL SERVICE OF THE HUNGARIAN ACADEMY OF SCIENCES recorded the opening of the pileus. Exposures were taken every 3 or 5 min (exposure time 20 s) throughout 70 h. The temperature of the room was 20 °C and the relative humidity 85%. The photos were then evaluated with a Film Motion Analyzer, manufactured by CAMERA SERVICE *Co.*, NISSËI SANGYO *Co.* Japan. Results obtained were plotted against the observation period (storage period) of mushrooms (KOVÁCS & ZUKÁL, 1977).

1.5. Determination of the weight of gills

All the gills were collected from 3×5 mushroom caps and weighed.

1.6. Study of spores

In the course of the experiments, the mushrooms were kept in a position to prevent shedding of the spores. From time to time, all the gills of 5 mushrooms were collected, of this 3×1 g gills were placed in the 0.04% solution of *Tween* 80. The spore count was determined in a *Bürker* cell after 24 h. The spores were counted in 25 large *Bürker* cells (0.004 mm^3). A phase contrast microscope was used (enlargement 40×10) in the study. The number of spores in 1 cm^3 was illustrated in the Figures. This method served to suspend the spores which were mature enough to separate from the basidium during the 24 h incubation period. The spores thus obtained were considered mature. The increase in the spore count permitted drawing conclusions as to the maturity of mushroom.

2. Results

2.1. Dynamics of cap opening in Agaricus bisporus *during storage subsequent to picking*

Figure 1 shows cap opening of some mushrooms. These results were taken from the evaluation of the filmshots. Opening is not a uniformly continuous process, it is periodical. Five to ten-h rapid opening periods alternate with 10–15 h periods of stagnation.

Figure 2 shows the frequency distribution as a function of time in mushrooms with closed cap and opened to varied width.

As can be seen, the number of mushrooms of closed cap diminishes linearly with time (Graph A). Afterwards, due to fractional opening, the situ-

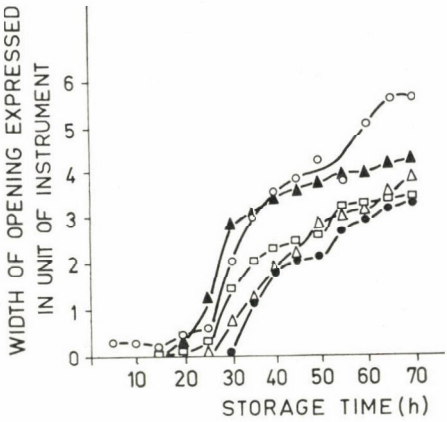


Fig. 1. Opening of the cap of some mushrooms based on the film made by the RESEARCH FILM STUDIO OF THE INSTRUMENT AND MEASUREMENT TECHNICAL SERVICE OF THE HUNGARIAN ACADEMY OF SCIENCES (Exposures were taken every 5 min during 70 h automatically, time of exposure 20 s. The films were evaluated on the FILM MOTION ANALYZER OF CAMERA SERVICE Co., NISSËI SANGYO Co., Japan)

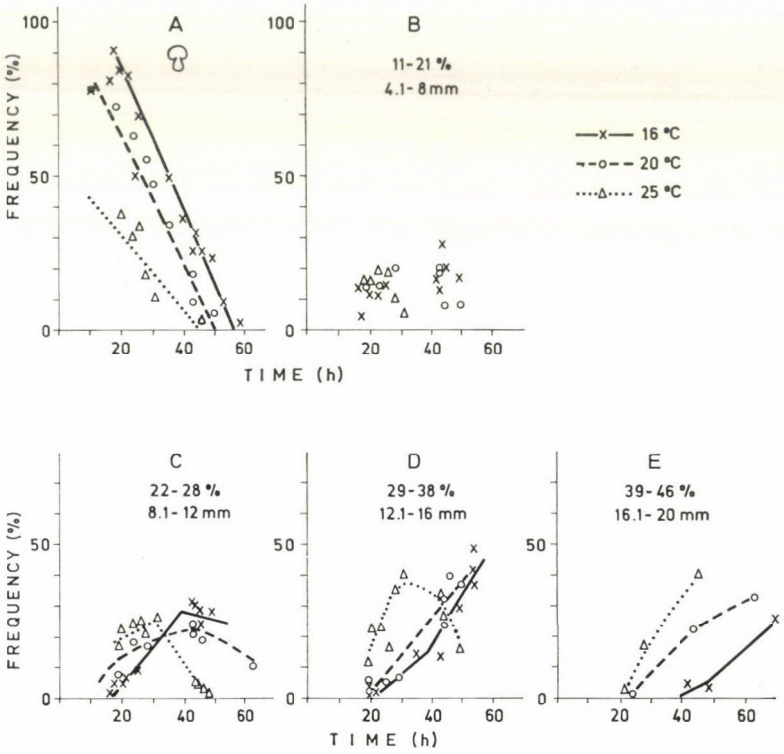


Fig. 2. Frequency distribution of mushrooms with closed cap (A), and opened to various width (B, C, D, E) as a function of time, based on several mushroom populations, at temperatures 16, 20 and 25 °C, resp. (Evaluation was based on data obtained by individual measurement of 1255 mushrooms.) mm: absolute value of cap opening; %: width of the opening related to the initial diameter of the cap

ation changes. It appears that opening rate is uniform up to 12 mm opening (*cca.* 11–28% relative opening). This is shown by the fact that, in these ranges of opening, mushrooms do not accumulate, but enter continuously the next range of opening. Thus in the range of 12.1–20 mm (relative opening of 29–47%) opening occurs more rapidly, at least at 16 and 20 °C, since the frequency increases uniformly with time. At 25 °C the same phenomenon appears with openings of 16–20 mm. At this temperature caps are opening wider.

2.2. Relationship between spore count and width of cap opening

Spore formation was found to start while the caps were still closed. The beginning of opening coincided with the increase of spore count (Fig. 3). The average value of opening (x) at the beginning of opening is marked in the Figure. The standard deviation around the mean is also shown.

In Fig. 4 the microscopic photographs of the spores of mushrooms of different opening width are shown. As seen in the photograph with increasing width of opening the spores become darker. Darkening of the spores is a symptom of maturation.

2.3. Spore count as affected by irradiation

Subsequent to radiation treatment not only the caps remain closed but the development of gills stops, too, thereby inhibiting the growth of spores as well (Table 1, Fig. 5).

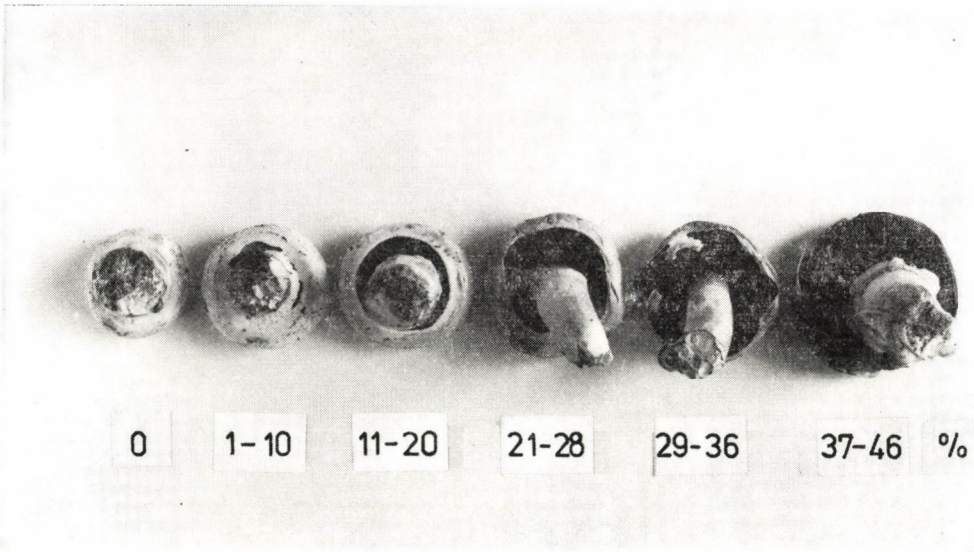
As can be seen in Fig. 5, the spore count increased with storage time. Already 1 kGy exerted an inhibiting effect on spores and from the third day

Table 1

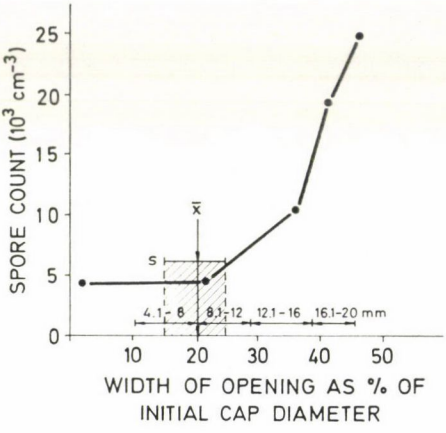
Proportion of the flesh to the gills in mushrooms as affected by radiation treatment

(at 14–16 °C and 80–90% RH)

Sample	g gills per 100 g mushroom	Proportion of gills to mushroom flesh	Increase of gills (%)
0 day 0 kGy	5.6	18.0	
2 days 0 kGy	9.8	10.2	75.0
6 days 0 kGy	11.6	8.6	107.1
0 day 2.5 kGy	5.6	18.0	
2 days 2.5 kGy	6.2	16.0	10.7
6 days 2.5 kGy	6.4	15.6	14.3



a)



b)

Fig. 3. a) The mushrooms at the top of the Figure belong to different ranges of opening. b) Change in the spore count as a function of the width of opening (at 14–16 °C, 80–90% RH). $\bar{x}$: average value; mm: width of the opening in mm

no increase was observed in the spore count. Inhibition was increased by treatment with 2.5 kGy.

In Fig. 6 the spores of mushrooms given different treatments are shown on the 1st and 6th day of storage. The spores of radiation-treated mushrooms differ in number and in refractive index from the untreated ones while neither the number, nor the refractive index of the irradiated spores changed during storage.

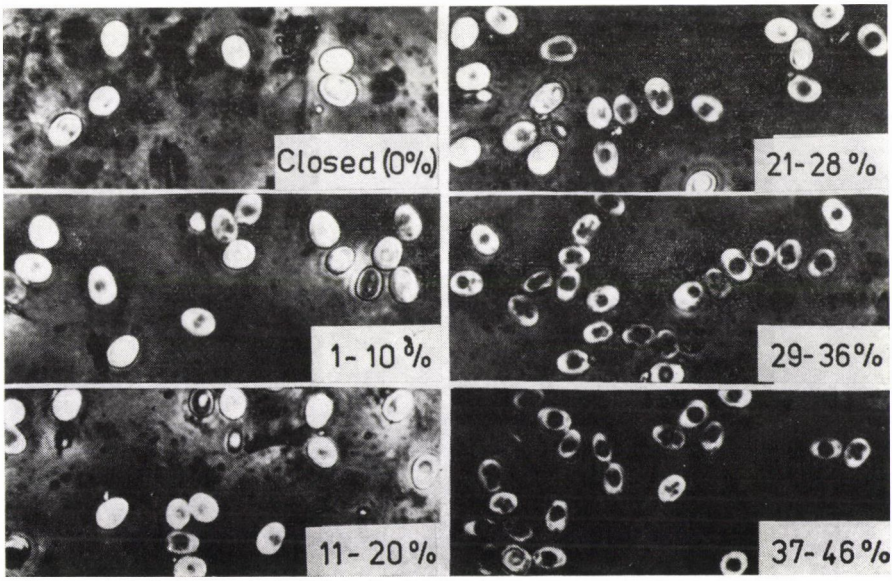


Fig. 4. Microscopic picture of the spores of mushrooms of different openings (Gill concentration in the suspension: 100 g l^{-1})

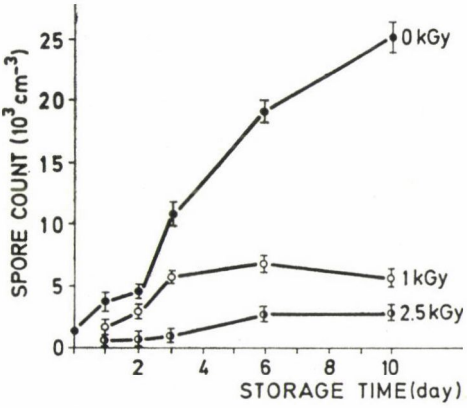


Fig. 5. Spore count as a function of radiation dose and storage time (at 14–16 °C; 80–90% RH)

According to data in the literature morphological changes in mushrooms after picking are regulated by the gills (GRUEN, 1963, 1967). CRAIG and co-workers (1977) found that during the opening of the cap of *Agaricus bisporus* growth is most intense in the top part of the stipe. They assume also growth by division. Ionizing radiation retards growth, opening of the cap, development of gills and stipe in *Agaricus bisporus*. Discontinuous opening of the

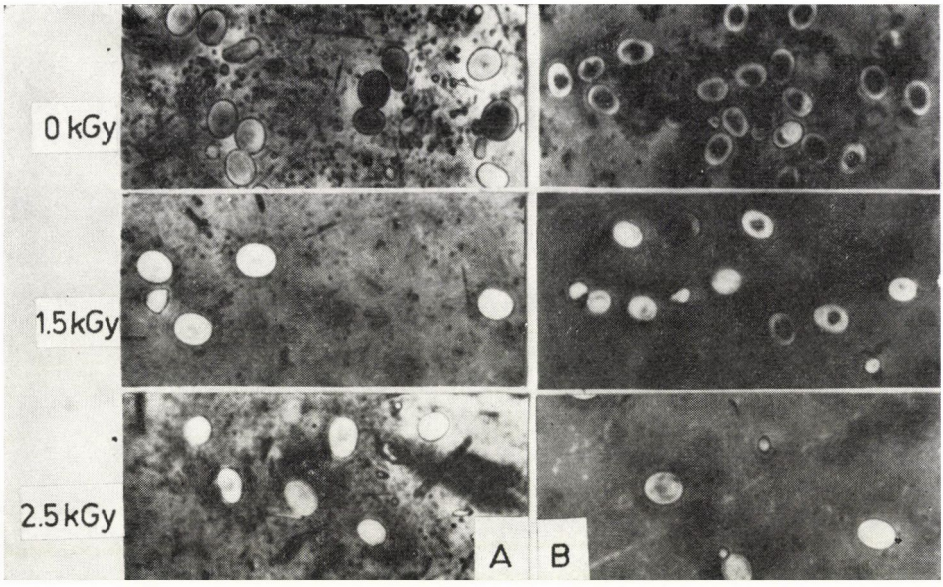


Fig. 6. Spores of mushrooms treated with different radiation doses on the 1st (A) and on the 6th (B) day of storage (Gill concentration in the suspension: 100 g l^{-1})

cap is most likely connected with maturation and shedding of the spores. The periodical character was shown in such important biochemical processes as the concentration changes of DNS. The discontinuos character was proven in the development of the fruiting body and it was shown to be in close correlation with its morphological changes (KITAMATO *et al.*, 1974). The discontinuous character in the opening of mushroom caps (alteration of rapid and slow periods) is connected in the slow phase with biochemical changes, while the rapid phase may be due to changes in the turgor in the cells (HAAR & KRAMER, 1970; ROCKETT & KRAMER, 1974).

It is assumed that the lower number of spores isolated from radiation treated mushrooms may be traced back to the inhibition of the maturing of spores or of spore formation at the level of division. Further experiments are necessary to elucidate this problem. Germination of spores is substantially inhibited by treatment with 0.16 and 0.64 kGy (KORONCZY & STUBNYA, 1974). These authors treated spores in powder form.

From the point of view of the food industry, inhibition of gill and spore number growth is important because dark gills and spores affect disadvantageously the processed product.

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RATING SCALES IN THE SENSORY ANALYSIS OF FOODSTUFFS

I. METHODOLOGICAL CONSIDERATIONS

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Sensory analyses are important methods for product development, for quality control, and to evaluate consumer preference. The importance of sensory evaluation lies in the fact that no instrument can perceive, analyse, integrate and interpret a large number of sensations at the same time.

The most important type of sensory methods are rating scale methods because of their wide range of application and their apparent simplicity. Also, the mathematical-statistical evaluation of data gained from rating scales is considered to pose no problems.

The nomenclature for rating scale methods comprises terms such as: grading, rating, scoring, scaling. The BRITISH STANDARDS INSTITUTION (1975) defines these methods as follows: "Rating (deprecated scaling): A method of classification into categories on an ordered scale. Scoring (deprecated grading): A form of rating using a numerical scale. The number used (scores) bear meaningful mathematical relationship. Grading (deprecated scoring): Classification of a product for quality by selected assessor(s) on the basis of one or more attributes."

The principle of a rating scale is to create the impression of a continuum related to some undimensional concept and provide the judge a ready means to locate an object in relative position on that continuum (AMERICAN SOCIETY FOR TESTING AND MATERIALS, 1968).

PLANK (1943) was of the opinion that a fundamental assumption of each rating scale is that the correlation between degree of quality and corresponding grade may be expressed by a straight line.

GUILFORD (1954) classified commonly used rating scales into five broad categories: numerical scales, graphic scales, standard scales, cumulated points, and forced choice. All of these scales require the assignment of manifestations of a given property by sensory judgements along an unbroken continuum or in ordered categories along the continuum, after which numbers are assigned. The scales differ in the operations of placement of manifestations of a given property, in the kind and number of aids or cues, and the fineness of discrimination demanded by the judge.

Sensory evaluation of foodstuffs by means of rating scales may be carried out by either methods with structured scales or methods with unstructured scales.

1. Methods with structured scales

– Graphic scales: are represented by a straight line divided into segments. The ends must be defined by contrary terms of a measurable property such as "more – less".

– Numerical scales: are graphic scales but with numbers assigned to the segments.

– Verbal scales: are graphic scales but with brief written statements assigned to the segments.

– Scales of standards: are graphic scales where all or at least a part of the segments are represented by samples of material.

Of main concern for all above methods is the type of scale used to measure manifestations of a given property. The type of scale may hinder or even prevent mathematical-statistical evaluation of data. STEVENS (1946) defined measuring in its broadest sense as the assignment of numbers or manifestations to a property of an object according to given rules. Different rules lead to different kinds of measurement. When a scale is designed, the theoretically possible manifestations of a property have to be considered. The process of measurement then represents recognition of an observed manifestation as one of the theoretically possible manifestations (PFANZAGL, 1971). According to NEWMAN (1974), "a theory of measurement is the logical structure which makes it possible to express psychological (more generally, scientific) facts in quantitative terms".

Depending on the type of scale (nominal, ordinal, interval or ratio scale), the data gained may be subjected only to permissible, specific statistical procedures. Quite frequently this fact is not considered when data gained from rating scales are subjected to mathematical-statistical analyses. For foodstuffs it may be supposed that ordinal scales are interval or ratio scales, but their correct homomorphic projection on the metric scale is still unknown (STREULI, 1967).

The points on a rating scale should be equidistant. If this is impossible, a clear succession of points on the scale should be ensured. The number of graduations on the scale depends on the number of intervals a judge can distinguish. It is essential that all judges define the criterion alike. The number of graduations is further to be adjusted according to the probable extent of variation which may occur when evaluating a certain foodstuff.

Investigations carried out by CARLIN and co-workers (1954) tried to gain some basic information on the effectiveness of different graduated scales (0–5, 0–10 and 0–100). The 0–100 scale proved to be as effective as the 0–5 scale, in some instances even superior. The 0–10 scale was compared to the 0–5 scale and proved to be more effective, however, this scale was not compared to the 0–100 scale.

ÖRSI (1973) showed that the number of intervals on a scale influences to a great extent the distribution of data. Data gained from a scale with only a few intervals show an asymmetric distribution. The asymmetry in distribution is due to the logarithmic connection (as defined by the theorem of WEBER and FECHNER), existing between the concentration of the component which predominantly influences a specific characteristic value of quality, and the thereby induced sensory perception. Applying the distribution theorem, one may calculate the number of sensory points from the distribution of the characteristic value of quality if the latter is presupposed to be normally distributed. The function of frequency derived from these equations is similar to the function of frequency of a normal distribution. The deviation is expressed in an exponential term which interprets the asymmetric distribution. The exponential term diminishes as the sensory point scale lengthens which means that also the asymmetry, *i.e.* the deviation from a normal distribution, diminishes. This fact makes it advisable to use scales with more than ten intervals.

CLONINGER and BALDWIN (1976) investigated data from nine previous sensory studies utilizing scales varying from 5 to 15 points and also gained asymmetrically distributed data. They also normalized data by calculating z-values to estimate the position of categories on the psychological continuum. In case of the 5-point scale, normalized data were almost equidistant. However, flavour data from 9- and 15-point scales showed a central tendency.

Already back in 1960 HARRIES stated that the validity of a scoring test, beside other parameters, depends also on a scale which produces data showing a reasonable distribution. But despite HARRIES' statement and despite the studies of ÖRSI (1973) and CLONINGER and BALDWIN (1976), there are still recommended rating scales with 3 (5) to 9 (11) intervals, as for instance: PAULUS and co-workers (1979), JELLINEK (1979), ROBINSON (1979). The mentioned studies employ parametric methods for mathematical-statistical evaluation of data, presuming that scores are normally distributed, categories are equally spaced, and that scores are proportional to the variation in the property being measured.

Verbal scales further include semantic problems. It is obvious that linguistic differentiation between various manifestations of a property does not suffice to describe the structure of a property accurately and systematically. Scientific analysis necessitates a finer graduation than provided by language.

STEVENS and GALANTER (1957) showed that rating scales lack the internal consistency desired in quantitative studies. This lack is due to the fact that a judge's ability to discriminate is better at the lower end of the sensory continuum. One may, however, directly assess subjective magnitude for perceived sensory properties.

TARVER and SCHENCK (1958) used rating scales in storage studies and found these scales tending to drift or to change in meaning, depending on time and on judges, unless they are anchored to a reproducible objective scale. It was suggested that the use of standards at various points in the scale would present a means to minimize panel variability. Even though this is a very plausible suggestion, it would pose problems barely surmountable if applied in the food industry because of the complex nature of foodstuffs.

2. Methods with unstructured scales

BATEN (1947) conducted comparison studies concerning palatability of some varieties of apples and pears. He applied a 7-point-rating-scale and a rating method with unstructured scale respectively for sensory evaluation. The unstructured scale consisted of a 6 inches long horizontal line, the left end marked "very poor" and the right end marked "excellent". Judges rated by drawing a vertical line across the horizontal line at the point representing their judgement. After the test, the horizontal line was divided into six units and the markings were transformed into numerical values. Most of the judges preferred to work with the 7-point rating scale, but the method with the unstructured scale led to larger t-values when comparative mathematical-statistical investigations were carried out. Also BYER and SALETAN (1961) used an unstructured scale for beer assessment. This scale was a 5 inches long vertical line. For each judge, a trend-line graph was set up which showed his rising or falling ratings for the successive tastings.

Own practical work with common scaling methods resulted in three categories of data: On the first and largest category, parametric methods were applicable. The second category of data allowed to apply parametric methods only after proper transformation of data. The third category could not at all be evaluated by means of mathematical-statistical procedures because of lack of distribution of data. These difficulties led to theoretical studies concerning structure and number of intervals of a scale and to the construction of an unstructured scale in 1971. The scale is represented by the diagonal, approximately 213 mm long, leading from the lower left corner to the upper right corner of a square (side length 150 mm). Each judge records his evaluation by making a line across the diagonal at the point that best reflects his perception of the manifestation of a given quality property. Depending on the investigated quality property, the lower left end of the diagonal represents the minimum and the upper right end the maximum manifestation of the property. After the test, the diagonal is superimposed by a matching overlay, divided into 100 units, to assign a number between 0 and 100 to each rating. The resulting numerical values may then be analysed.

This scale has been thoroughly tested in numerous and extensive sensory analyses and proved to yield data which may be interpreted by means of parametric methods without any transformation. This fact is demonstrated in published studies, as for instance: WEISS and co-workers (1972): Apple juice and pear nectar; ZAUSSINGER and co-workers (1975): Staling of bread; KNORR and co-workers (1976): Dried protein products of potato juice; and WEISS (1981): Selection of sensory judges.

Three years later, STONE and co-workers (1974) at the Stanford Research Institute constructed an unstructured rating scale, based on similar considerations. This scale is called "QDA" (*Quantitative Descriptive Analysis*) and consists of a 6 inches long horizontal line. This line is provided with two anchor points, located 0.5 inches from the ends of the scale. Both ends of the scale are assigned descriptive terms such as: weak – strong, soft – hard, flat – bubbly. A third anchor point may be located at the mid-point of the scale, assigned the descriptive term "moderate". After the test, the scale is divided into 60 units and the resultant numerical values are analysed. This *QDA*-method was successfully applied on sensory evaluation, for example of beer (MECREDY *et al.*, 1974) and wine (WING-O-KWAN and KOWALSKI 1980).

The described advantages allow the prediction that rating methods with unstructured scales will become essential tools in sensory evaluation of foodstuffs and also with regard to applied multivariate analyses.

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RATING SCALES IN THE SENSORY ANALYSIS OF FOODSTUFFS

II. PARADIGMATIC APPLICATION OF A RATING METHOD WITH UNSTRUCTURED SCALE

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The sensory quality property *odour specificity* of six differently treated ciders was assessed by means of a rating method with unstructured scale. Detailed steps of calculation demonstrate the correct procedure when interpreting data by means of mathematical-statistical tests.

Production and consumption of cider and perry increased considerably during the last ten years in Austria. The technology of these products affords preventive steps to avoid turbidity in bottled wines. Turbidity is partly caused by proteins, and, therefore, these are eliminated by *HTST* treatment and/or by bentonite treatment, but such treatments also eliminate some desirable components of the wines. Therefore, it seemed reasonable to decompose proteins – contained in ciders and perries – by means of proteolytic enzymes, bearing in mind, however, that some proteases produce fractions with a specific taste depending upon the substrate (HIGGINBOTHAM *et al.*, 1977). Consequently, values of sensory quality properties (odour, taste) may be changed after enzymatic treatment.

Studies were carried out to investigate whether a treatment of apple juices or ciders with proteolytic enzymes results in stabilized wines (WEISS *et al.*, 1980) and whether thereby values of sensory quality properties are changed significantly. The present paper concerns itself in a paradigmatic way with the sensory quality property *odour specificity*.

1. Materials and methods

1.1. Material

Apple juice was produced on industrial scale from apples of the variety *Kaiser Wilhelm*. The juice was subjected to treatments as listed in Fig. 1. Commercial proteolytic enzymes were used in the form of *Biocon-Pilz-Protease AP* (BIOCON GmbH., D-8201 Kolbermoor, FRG) in the amount of 5 g hl^{-1}. The source organism for this compound is *Aspergillus oryzae*. These proteases are of high efficiency at lower pH-values (pH-optimum 2.4–3.7).

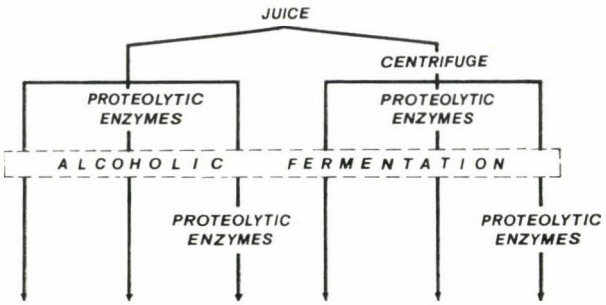

Fig. 1

1.2. Sensory analysis

Sensory evaluation of the quality property *odour specificity* was carried out by means of a rating method with unstructured scale (Fig. 2) (WEISS *et al.*, 1972; WEISS, 1981). The scale consists of a square (side length 150 mm) and its diagonal from the lower left end to the upper right end. The lower left end was assigned the most negative manifestation of the quality property *odour specicifity*, namely *off-odour*, whereas the upper right end was assigned the most positive manifestation of this quality property, namely *excellent odour*. The diagonal was chosen as an indicator for the upward trend in the manifestations of the quality property under consideration. For each judgement, the judge had to draw a little vertical line across the diagonal at the point that best reflected the judge's perception of the manifestation of the quality property *odour specificity*. One sitting comprised seven judges, six samples and five replications.

The forms used permitted notations of a series of six judgements. Sequence of the six samples and their codification were established by means of random

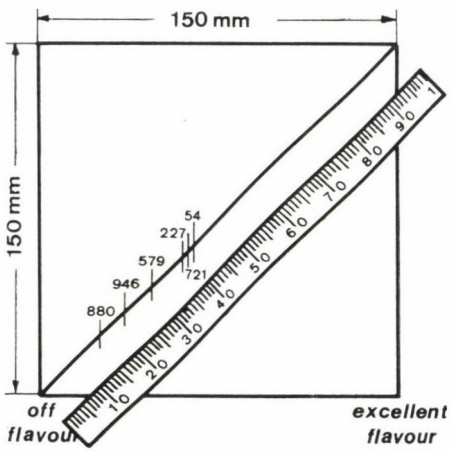

Fig. 2

digits (MENGES *et al.*, 1973). After the sitting, the notations on the forms were transformed into values, using an overlay of the same length as the diagonal, but divided into 100 units.

1.3. Mathematical-statistical analysis

To allow comparison of sensory judgements of two or more products by means of parametric tests to determine whether they differ significantly, it is necessary to establish whether the values are normally distributed and show homogeneity of variance.

The values for one sample may be subjected to the χ^2-test of goodness to fit to establish whether they are normally distributed. This means that their empirical distribution is compared to the normal distribution. Even though this test would request an unlimited number of samples, it may also be applied to a limited number of samples ($n \gtrsim 10$), containing only few classes ($k \gtrsim 5$) (SACHS, 1978). Rating methods with structured scales (scoring) mostly do not provide the postulated number of classes. DAVID *et al.* (1954) designed a test which allows to determine quickly whether or not the investigated values are normally distributed. The quotient from range and standard deviation is compared with table values (PEARSON *et al.*, 1964). If the limits of the table values are reached, surpassed or are not reached, then the hypothesis of normality is to be rejected at the corresponding level of significance. Critical values at the 0.1 significance level are to be used for a decision between applicability or rejection of parametric tests (SACHS, 1978).

Homogeneity of two or more variances may be investigated – assuming sample groups of equal size – by means of a test described by HARTLEY (1950a, b). Mostly, however, sample groups are not of equal size. Then it is more advantageous to apply the test designed by BARTLETT (1937) which, on the one hand has a broader range of applicability, but, on the other hand, requests data following strictly a clear normal distribution.

If the test value χ^2 reaches or surpasses the critical value corresponding to the requested significance level, then the null-hypothesis has to be rejected.

If the values are normally distributed and if homogeneity of variance is established, a quantitative investigation of parameters may be conducted by means of an analysis of variance.

If the estimated F-value is larger than the table value at a given level of significance, a multiple comparison by means of the modified least-significant-difference-test ($L.S.D.$-test) may be conducted. Arithmetic mean values of equally sized sample groups are ranged according to falling size. Then it is investigated whether adjoining arithmetic mean values show a larger difference (Δ) than the least significant difference.

Table 1

Sensory judgement of the quality property odour specificity

Judge	Samples and replications																											
	A					B					C					D					E					F				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
a	39	40	41	38	39	41	38	39	40	42	43	45	44	43	44	17	15	16	17	16	31	30	30	29	30	24	22	23	24	22
b	43	43	37	40	41	46	41	47	44	36	44	44	45	41	46	11	12	12	15	11	27	28	28	27	31	23	22	21	21	20
c	41	36	40	42	34	44	39	43	45	37	47	47	50	46	46	20	20	17	10	12	27	23	34	27	29	23	22	25	25	22
d	33	37	37	40	35	39	38	42	38	42	48	47	49	49	49	14	14	15	15	15	27	32	28	27	30	17	20	21	22	22
e	43	43	44	46	44	43	45	41	36	40	47	50	46	46	50	18	20	19	15	13	27	25	34	26	28	18	23	26	23	26
f	40	43	42	45	44	43	45	46	40	40	44	51	48	49	52	18	20	19	15	13	31	30	30	29	30	21	23	25	24	23
g	42	41	39	40	38	41	42	39	41	41	45	44	45	44	40	10	19	17	8	15	33	26	31	30	32	25	18	27	23	24
$\bar{x}$	40.29					41.26					46.23					15.23					29.06					22.57				
$\pm s$	3.12					2.86					2.80					3.21					2.45					2.27				
s^2	9.74					8.20					7.83					10.30					5.80					5.13				
R	13					11					12					12					11					10				

Table 2

χ^2-test of goodness of fit for sample A

x	B	x^2	$B \cdot x$	$B \cdot x^2$	$x - \bar{x}$	$z = \frac{x-\bar{x}}{s}$	$f(z)$	$f(z) \cdot K$	E	$B-E$	$(B-E)^2$	$\frac{(B-E)^2}{E}$
33.5	2	1122.25	67.0	2 244.50	−6.8	2.18	0.0371	0.834	3.574	+0.426	0.181	0.051
35.5	2	1260.25	71.0	2 520.50	−4.8	1.54	0.1219	2.740				
37.5	5	1406.25	187.5	7 031.25	−2.8	0.90	0.2661	5.982	5.982	−0.982	0.964	0.161
39.5	9	1560.25	355.5	14 042.25	−0.8	0.26	0.3857	8.671	8.671	+0.329	0.108	0.012
41.5	7	1722.25	290.5	12 055.75	+1.2	0.39	0.3697	8.311	8.311	−1.311	1.719	0.207
43.5	8	1892.25	348.0	15 138.00	+3.2	1.03	0.2347	5.276	7.499	+2.501	6.255	0.834
45.5	2	2070.25	91.0	4 140.50	+5.2	1.67	0.0989	2.223				
	35		1410.5	57 172.75					34.037	+0.963		1.265

$$\bar{x} = \frac{\Sigma B \cdot x}{n} = \frac{1410.5}{35} = 40.30\,, \qquad s = \sqrt{\frac{\Sigma B \cdot x^2 - \frac{(\Sigma B \cdot x)^2}{n}}{n-1}} = \sqrt{\frac{57172.75 - \frac{1989510.2}{35}}{34}} = 3.114\,, \qquad K = \frac{n \cdot b}{s} = \frac{35.2}{3.114} = 22.48$$

2. Results and discussion

Table 1 shows the values for the quality property odour specificity resulting from the sensory evaluation of six ciders, carried out by seven qualified judges with five replications.

2.1. Test of normal distribution

The χ^2-test of goodness to fit served to determine whether the values are normally distributed. The detailed calculation steps follow, using the values of sample A (Table 2):

1. Formation of classes: Always two adjoining values were pooled into a class (33 + 34, 35 + 36 . . . 45 + 46); the width of classes (b) is therefore 2.

2. Observed frequency (B) per class.

3. Calculation of mean values of classes: x (33.5, 35.5 . . . 45.5)

4. If the $f(z) \cdot K$-values per class were below 4, then adjoining classes were pooled.

The calculated χ^2-value is 1.27 (1.265) and by far does not reach the χ^2-table value (α 0.1) which is 4.61. Therefore, the hypothesis of normality may be accepted.

Table 3 shows that for all six samples the values calculated for χ^2 were far below the corresponding χ^2-table values (α 0.1). This means that all values of all samples are normally distributed.

The test of normal distribution as described by DAVID and co-workers (1954) – the critical values for $n = 35$ and $\alpha = 10\%$ are 3.70–4.84 – led to the same result (see Table 3).

The following theoretical example demonstrates the apparent advantage which the described rating method with unstructured scale offers as compared to a 5- resp. 9-point scale when it comes to mathematical-statistical evaluation of data: Supposing that the same judges had evaluated the same samples with five replications and had differentiated in the same manner, a 5-point scale (1 ≙ 0–20, 2 ≙ 21–40, 3 ≙ 41–60, 4 ≙ 61–80 and 5 ≙ 81–100 of the values of the unstructured scale) would have yielded values as shown in Table 4, and a 9-point scale (1 ≙ 0–11, 2 ≙ 12–22, 3 ≙ 23–33, . . . 9 ≙ 89–99 of the values of the unstructured scale) would have yielded values as shown in Table 5.

The results would clearly show that the hypothetical application of a 5-point scale would not have permitted a mathematical-statistical evaluation of data by means of parametric methods because values for samples A, B, C and F would not be normally distributed and values for samples D and E would not show any distribution.

The results as shown in Table 5 also indicate that data gained hypothetically from a 9-point scale would not be normally distributed. All values for

Table 3

The results of the test of normality

Sample	χ^2	χ^2-table value	R/s
A	1.27	4.61	4.17
B	0.87	2.71	3.84
C	1.81	4.61	4.29
D	1.59	4.61	3.74
E	0.35	2.71	4.49
F	0.40	2.71	4.41

Table 4

Hypothetical data gained by means of a 5-point scale

Points	Samples					
	A	B	C	D	E	F
1	—	—	—	35	—	5
2	18	14	1	—	35	30
3	17	21	34	—	—	—
4	—	—	—	—	—	—
5	—	—	—	—	—	—
$\bar{x}$	2.49	2.60	2.97	1.0	2.0	1.86
$\pm s$	0.507	0.497	0.169	—	—	0.355
R/s	1.97	2.01	5.92	—	—	2.82

Table 5

Hypothetical data gained by means of a 9-point scale

Points	Samples					
	A	B	C	D	E	F
1	—	—	—	5	—	—
2	—	—	—	30	—	16
3	1	—	—	—	33	19
4	32	29	11	—	2	—
5	2	6	24	—	—	—
6	—	—	—	—	—	—
7–9	—	—	—	—	—	—
$\bar{x}$	4.03	4.17	4.69	1.86	3.06	2.54
$\pm s$	0.296	0.382	0.471	0.355	0.236	0.506
R/s	6.76	2.62	2.12	2.82	4.24	1.98

R/s would not fit between the 10% limits (3.70–4.84) with the exception of sample E. Therefore, a direct mathematical-statistical interpretation by means of parametric tests would not be applicable.

Neither hypothetical values from the 5-point scale, nor the 9-point scale could be subjected to the χ^2-test of goodness to fit because the minimum number of classes ($k \gtrsim 5$) could not be reached. In practical work it may also be difficult to meet this requirement when working with qualified judges.

2.2. *Homogeneity of variance*

The *Bartlett*-test was used to investigate homogeneity of variance, based on data as shown in Table 6.

Table 6

Data for the Bartlett-*test*

Sample	s_i*	s_i^2	$\lg s_i^2$
A	3.121	9.739	0.98851
B	2.863	8.197	0.91365
C	2.798	7.829	0.89371
D	3.210	10.299	1.01280
E	2.449	5.997	0.77793
F	2.266	5.134	0.71046
		47.195	5.29706

* see table 1.

Because the six sample groups are of the same size ($n_0 = 35$), χ^2 may be calculated using a simplified equation (SACHS, 1978):

$$\chi^2 = \frac{1}{c}\left[2.3026 \cdot k \cdot (n_0 - 1)\left\{\lg s^2 - \frac{1}{k}\sum_{i=1}^{k} \lg s_i^2\right\}\right]$$

$$c = \frac{k+1}{3k(n_0 - 1)} + 1$$

$$s^2 = \frac{1}{k}\sum_{i=1}^{k} s_i^2$$

Introducing the values of Table 5, further $k = 6$ (A, B, C . . . F) and $n_0 = 35$ into the equation, the resulting values are:

$$s^2 = \frac{1}{6} \cdot 47.195 = 7.866 \qquad \lg s^2 = 0.8957$$

$$c = \frac{6+1}{3 \cdot 6 \cdot 34} + 1 = 1.011$$

$$\chi^2 = \frac{1}{1.011} \cdot \left[2.3026 \cdot 6 \cdot 34 \cdot \left(0.8957 - \frac{1}{6} \cdot 5.297 \right) \right] = 5.95$$

The χ^2-table value $P \geq 0.05 = 11.07$ is much larger than 5.95. Therefore, the null-hypothesis is not rejected which confirms homogeneity of variance.

2.3. Analysis of variance

Analysis of variance may be carried out because sensory data for each cider follow a normal distribution and because homogeneity of variance was established. Results are shown in Table 7.

Table 7

Analysis of variance

Source of variation	*d.f.*	*s.s.*	*M.S.*	*F*-ratio
Between samples	5	25 707.07	5 141.41	651.20
Within samples	204	1 610.58	7.90	
Total	209			

The calculated F-value considerably surpasses the F-table value 200, α 0.01 = 3.11. Therefore, the null-hypothesis (conformity of the six mean values) has to be rejected at the 1% level.

2.4. Multiple comparisons

Because of rejected null-hypothesis, the least significant differences, at a given level of significance, may be calculated, using the following equation (SACHS, 1978):

$$\text{L.S.D.} = t_{n-k,\alpha} \cdot \sqrt{\frac{2}{n_0} \cdot s^2}$$

$$\text{L.S.D.} = t_{204,\alpha} \cdot \sqrt{\frac{2}{35} \cdot s^2} = t_{204,\alpha} \cdot 0.670$$

$$\text{L.S.D.}_{\alpha\,0.05} = 1.972 \cdot 0.670 = 1.32$$

$$\text{L.S.D.}_{\alpha\,0.01} = 2.601 \cdot 0.670 = 1.74$$

Table 8

Arithmetic mean values and differences between samples

Sample	$\bar{x}$	Δ
C	46.23	
		4.97
B	41.26	
		0.97
A	40.29	
		11.23
E	29.06	
		6.49
F	22.57	
		7.34
D	15.23	

The following Table 8 presents the arithmetic mean values (see Table 1) according to their sizes and their differences (Δ).

All differences, except for the difference cider B *vs.* cider A, are larger than 1.74 (L.S.D. α 0.01) which means that highly significant (α 0.01) differences exist between the ciders (with the exception of B *vs.* A) concerning the sensory quality property odour *specificity*. The data contained in Table 8 also permit to interpret the sizes of differences.

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ASSAY INTO THE VOLATILE AMINES IN TOMATOES AND TOMATO PRODUCTS

II. COMBINED GAS-CHROMATOGRAPHIC – MASS-SPECTROMETRIC INVESTIGATIONS, ESTIMATION OF THE VOLATILE AMINE CONTENT

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The extraction, enrichment and gas-chromatographic separation methods developed earlier (Madarassy-Mersich *et al.*, 1978) have been used along with mass spectrometry for the identification of the respective amines and the estimation of the volatile amine content in fresh tomatoes and tomato products.

Experiments were carried out under identical conditions with fresh tomatoes, tomato purée and powdered tomato. The presence of methyl-, dimethyl-, trimethyl-, ethyl-, ethyldimethyl-, n-propyl-, diethyl-, i-butyl-, n-butyl-, and two iso-pentylamines (2-methyl-butylamine and 3-methyl-butylamine) could be proved in all three samples. However, n-pentylamine could only be identified in tomato purée. The identity of these compounds, except that of n-butyl- and n-pentylamine, was further substantiated by mass spectrometric examinations as well.

The amount of volatile amines in the samples has also been assayed. The estimated total volatile amine content of fresh tomatoes is about 1 ppm. Two amines, i-butyl and i-pentylamine account for about 90% of this amount. Processing decreases the volatile amine content significantly. Tomato purée contained about half as much volatile amines as fresh tomatoes, while powdered tomato contained about a third of the volatile amine content of its raw material, tomato purée.

A method was described in Part I (Madarassy-Mersich *et al.*, 1978) for the extraction and gas-chromatographic separation of the volatile amines of fresh tomatoes and tomato products. Both retention time of the components on two different gas-chromatographic stationary phases and the so-called enrichment technique were used to identify the components. Out of the 13–16 components present in all three samples, 9 amines could be identified by this method. The aim of the present work has been the confirmation of the structures of the tentatively identified compounds and the determination of the so-far unknown structures by combined gas-chromatographic–mass-spectrometric (GC–MS) investigations, as well as the estimation of the volatile amine levels of tomatoes.

1. Materials and methods

The sample series used for the GC–MS examinations was identical with that used previously for the development of the experimental methodology (Madarassy-Mersich *et al.*, 1978). The gas-chromatograms of the volatile amine fractions extracted from fresh tomatoes, tomato purée and powdered tomato are shown in Fig. 1.

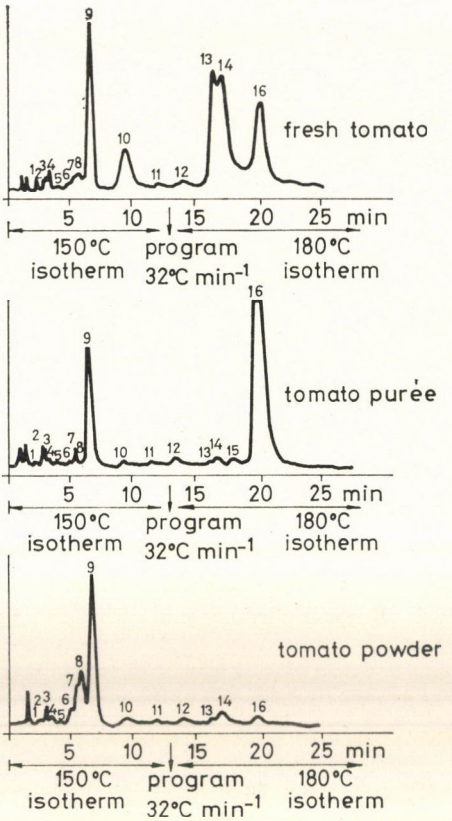

Fig. 1. Gas-chromatographic separation of volatile amines, 1976 batch. The temperature program is seen in the figure. Peaks: (1) methylamine; (2) dimethylamine; (3) unidentified; (4) ethylamine; (5) unidentified; (6) unidentified; (7) unidentified; (8) propylamine; (9) diethylamine; (10) i-butylamine; (11) butylamine; (12) unidentified; (13) i-pentylamine; (14) unidentified; (15) pentylamine; (16) unidentified; Gas chromatographic column: 3.6 m length, 2 mm inner diameter, stainless steel; Stationary phase: *Chromosorb* 103; Carrier gas velocity: 30 cm^3 min^{-1}; Injector temperature: 230 °C; Detector temperature: 230 °C

The volatile amine content of the samples was estimated only one year later. The origin of the samples and the methodology was identical, except for the quantity of the samples which was doubled. Thus, 12 000 g fresh tomatoes, 2 520 g tomato purée and 784 g powdered tomato were examined.

1.1. Identification of the components by combined GC–MS

The free amine samples were examined by a *Varian* MAT 111 GC–MS system. The separation conditions used in the present work were as similar to the previous conditions as possible. A 3.6 m long, 2 mm inner diameter, stainless steel column packed with *Chromosorb* 103 was used for the separations.

Helium at 30 $cm^3 \cdot min^{-1}$ was used for carrier gas. The injector and the detector were kept at 230 °C. The electron accelerating voltage in the ion-source of the mass spectrometer was set at 70 V. The chromatograms were recorded by the electron impact ionization detector (EIID) of the GC–MS system. (The sensitivities of the EIID and FID are comparable.)

In order to obtain reasonable mass spectra, the amount of the amine hydrochloride salt mixture, the distilled water used for its dissolution and the organic solvent used for the extraction of the free amines had to be increased. Best results were obtained with 20 mg amine salt mixture, 30 μl distilled water and 30 μl hexadecane solvent. Complete phase separation could be achieved by centrifugation. 20 μl organic phase were injected into the GC–MS for one separation.

The compounds were identified by evaluating their mass spectra and comparing them with literature reference spectra.

2. Results

2.1. Identification by GC–MS of the amine bases extracted from tomatoes

Identification of the components was relatively easy, because the fraction tested contained only amine bases and the previous enrichment experiments indicated at least a tentative structure for almost each peak.

The list of the compounds identified by their mass spectra, along with the *m/e* of their five most intense fragments are shown in Table 1.

Naturally, not only the five most intense peaks, but all significant peaks of the mass spectra were compared. When necessary, the intensity ratios of the neighbouring peaks were also calculated and compared with literature data. Positive structure assignment was made only when all the above parameters agreed with literature data.

The following conclusions can be drawn from the analysis of the data shown in Table 1.

The agreement of the two data series is excellent for methylamine, sufficiently good for dimethylamine, trimethylamine, ethylamine and ethyldimethylamine. The spectrum of n-propylamine is contaminated by the fragments of the only partially separated ethyl-dimethylamine. The reversal of the intensity ratios noted in the mass spectrum of diethylamine is due to the rapid change of the amine concentration during recording.

Since the order of the five most intense fragments are identical in the case of both the normal and the iso-butylamines, the ion intensity ratios of the most dissimilar peaks of the spectrum had to be used for their identification as shown in Table 2.

Table 1

Identification of the volatile amine bases of tomato by GC–MS

Number[a]	Name	Spectrum[b]		Five major peaks (*m/e* %[c])			
1	Methylamine	i^s	30 (100%)	28 (88%)	31 (56%)	29 (16%)	27 (16%)
		m	30 (100%)	28 (77%)	31 (60%)	29 (19%)	27 (16%)
2	Dimethylamine	i^s	44 (100%)	28 (68%)	45 (51%)	15 (20%)	42 (19%)
		m	44 (100%)	28 (51%)	45 (51%)	15 (27%)	42 (29%)
3	Trimethylamine	i^s	58 (100%)	42 (46%)	59 (39%)	15 (34%)	30 (18%)
		m	58 (100%)	42 (49%)	59 (39%)	15 (37%)	30 (20%)
4	Ethylamine	i^s	30 (100%)	28 (29%)	44 (20%)	45 (19%)	27 (13%)
		m	30 (100%)	28 (35%)	44 (35%)	45 (28%)	27 (25%)
7	Ethyl-dimethylamine	i^m	58 (100%)	42 (28%)	44 (25%)	73 (23%)	30 (13%)
		m	58 (100%)	42 (35%)	44 (32%)	73 (17%)	30 (17%)
8	n-propylamine	i^s	30 (100%)	28 (13%)	59 (8%)	27 (7%)	41 (5%)
		m	30 (100%)	20 (18%)	59 (13%)	27 (8%)	41 (7%)
9	Diethylamine	i^m	58 (100%)	30 (73%)	73 (34%)	44 (31%)	72 (20%)
		m	30 (100%)	58 (73%)	73 (30%)	44 (29%)	72 (12%)
		i^s	58 (100%)	30 (98%)	28 (37%)	27 (29%)	44 (29%)
		m	30 (100%)	58 (73%)	28 (27%)	27 (28%)	44 (29%)
10	i-butylamine	i^s	30 (100%)	28 (9%)	41 (6%)	27 (5%)	73 (5%)
		m	30 (100%)	28 (44%)	41 (27%)	27 (16%)	73 (24%)
13 14	i-pentylamine	i^s	30 (100%)	28 (8%)	27 (8%)	41 (6%)	39 (6%)
		m	30 (100%)	28 (20%)	27 (14%)	41 (17%)	39 (13%)

[a] Numbers correspond to peak numbers in Fig. 1
[b] i^s literature data, STENHAGEN et al. (1969)
i^m literature data McLAFFERTY (1973)
m sample spectrum
[c] *m/e* mass change ratio
% peak intensity relating to the base peak of the spectrum

Due to their partial gas-chromatographic separation and the lack of reference spectra the exact branching of the two i-pentylamines could not be established.

The amount of n-butylamine in the chromatogram of the amine fraction of fresh tomatoes was so low that a useful mass spectrum could not be obtained. Nor did this sample contain n-pentylamine, either.

Table 2

Evaluation of the mass spectra of n- and i-butylamines, analysis of a section of their mass spectra

m/e	n-butylamine		i-butylamine		Measured spectrum	
	%[a]	intensity ratio[b]	%[a]	intensity ratio[b]	%[a]	intensity ratio[b]
56	1.10	3.70	2.10	1.11	17.0	1.21
57	0.23	0.77	1.10	0.58	8.0	0.57
58	0.30	1.00	1.90	1.00	14.0	1.00
59	0.02	0.07	0.08	0.04	1.1	0.06

[a] Literature data, McLAFFERTY (1973).
[b] Intensity ratios relate to the intensity of *m/e* 58 peak

Our present knowledge relating to the amine bases of fresh tomatoes and tomato products are summarized in Table 3.

The compounds listed as unknown in Table 3 (Peaks 5, 6, 12 and 16) are not amines, as evidenced by their mass spectra. The most intense peak, Peak 16 is toluene. Toluene, which is easily separated from n-pentylamine under the experimental conditions, is an artifact, introduced by abs. ethanol used in large quantities for the washing of the amine salt precipitate. Depending on the actual batch of ethanol used its quantity fluctuated widely.

The other three peaks are also believed to be artifacts but their identity was not pursued any further.

2.2. Estimation of the volatile amine content

The gas chromatograms of the volatile amine fractions extracted from the three tomato samples are shown in Fig. 2.

The chromatograms substantiate the major conclusions of our investigations carried out in the previous year. Namely,

– the volatile amine level is highest in fresh tomatoes and lowest in the tomato powder sample

– the amounts of the i-butyl- and i-pentylamines differ most

– the amount of volatile amines decreases with increasing industrial processing (fresh tomatoes → tomato purée → tomato powder).

Neither the number, nor the quality of the compounds separated differed significantly.

The increased samples resulted in more intense chromatograms, only the diethylamine peak did not increase proportionally. Presumably, the higher loadings used in this year caused the incomplete separation of dimethyl-

Table 3

Volatile amines identified in the fresh and processed tomato samples

Peak number[a]	Compound	Estimated quantity[b]			Identification method[c]
		I	II	III	
1	methylamine	S	T	T	RT; ET; MS
2	dimethylamine	S	S	S	RT; ET; MS
3	trimethylamine	S	S	S	MS
4	ethylamine	S	T	T	RT; ET; MS
5	unknown	T	T	T	
6	unknown	T	T	S	
7	ethyl-dimethylamine	S	S	S	MS
8	n-propylamine	S	T	M	RT; ET; MS
9	diethylamine	L	L	L	RT; ET; MS
10	i-butylamine	M	T	S	RT; ET; MS
11	n-butylamine	T	T	T	RT; ET
12	unknown	S	S	S	
13	i-pentylamine	L	T	T	RT; ET; MS
14	i-pentylamine	L	S	S	RT; ET; MS
15	n-pentylamine	O	S	O	RT; ET
16	unknown	L	L	T	

[a] Peak number corresponds to numbering in Fig. 1
[b] I = fresh tomatoes
II = tomato purée
III = tomato powder
L = large amount
M = medium amount
S = small amount
T = trace amount
O = non-detectable
[c] RT = retention time
ET = enrichment technique
MS = mass spectrometry

and trimethylamine peaks which closely followed each other. Another, non-amine artifact peak could also be detected. The only significant difference between the results of the previous and the present year was the increase in the amount of the i-butyl- and i-pentylamines at the tomato purée sample.

These two amines, as will be discussed in a forthcoming paper, significantly influence the odour of the tomato purée. Since the raw material used this year was more ripe and of better quality, the increased amounts of these two amines are understandable.

In order to obtain a rough estimate of the volatile amine content of the tomato samples, another experiment was carried out using n-butylamine as internal standard. Since the samples contained only traces of n-butylamine,

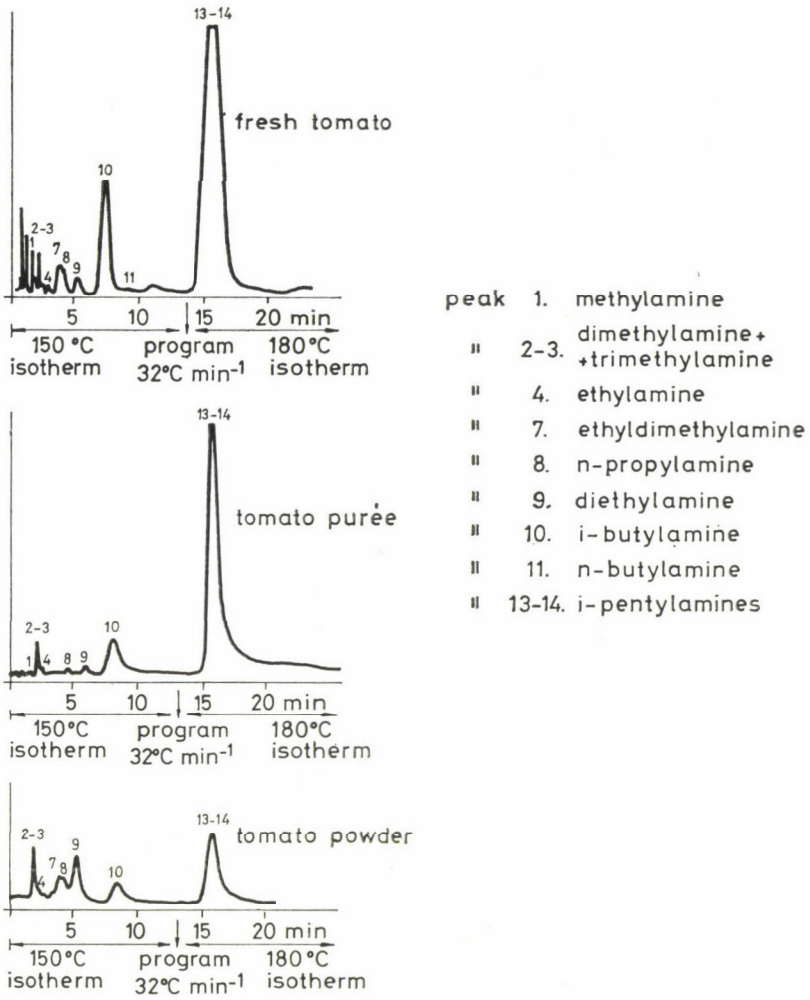

Fig. 2. Gas-chromatographic separation of volatile amines, 1977 batch. (For conditions of gas chromatography see Fig. 1)

and it eluted at the middle of the chromatogram, it seemed a suitable internal standard.

The chromatogram of the amine fraction of the n-butylamine-spiked fresh tomatoes is shown in Fig. 3.

The peak areas and their area percentages are summarized in Table 4.

It can be seen that 87% of the volatile amine content of fresh tomatoes is accounted for by the i-butyl- and i-pentylamines. Therefore, it seems reasonable to express the volatile amine content of the samples as i-pentylamine. Based on the initial sample amounts used, the concentration of the internal

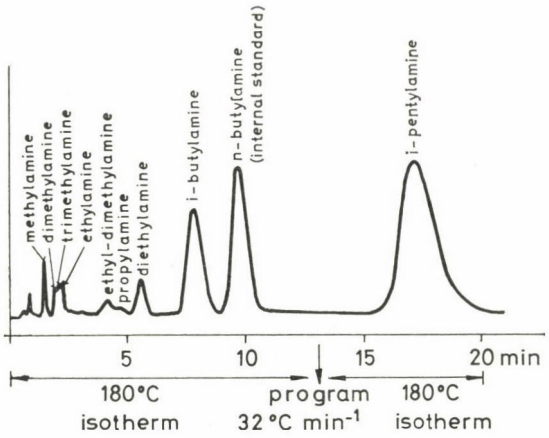

Fig. 3. The chromatogram of the amine fraction of the n-butylamine-spiked fresh tomatoes (For conditions of gas chromatography see Fig. 1)

Table 4

Estimation of the volatile amine content of fresh tomatoes

Name	Peak area (cm^2)	%
Methylamine	0.30	1.2
Dimethylamine	0.26	1.0
Trimethylamine	0.40	1.6
Ethylamine	0.61	2.4
Ethyl-dimethylamine	0.52	2.1
n-propylamine	0.24	1.0
Diethylamine	1.01	4.0
i-butylamine	4.64	18.5
Internal standard	(6.79)	—
i-pentylamine	17.06	68.2
Total	25.04	100.0

standard and peak areas, the volatile amine content of fresh tomatoes expressed as i-pentylamine is 0.2 mg kg^{-1}.

Due to certain losses during processing the volatile amine content of fresh tomatoes might be somewhat higher, but probably not higher than 1 ppm.

Since the quantitative determination of the volatile amine content is somewhat uncertain even in the case of the most concentrated sample, fresh tomatoes, quantitation has not been attempted for the tomato purée and powder samples. However, the decrease of the amine content could be followed

by the chromatograms. When the areas of the major peaks i.e. i-butyl- and i-pentylamines are compared with the peak areas in the fresh tomato sample taken as 100%, the respective values become 55% for tomato purée and 18% for tomato powder.

3. Conclusion

To summarize the results of this and the related former work (MADARASSY-MERSICH *et al.*, 1978) it can be concluded that a suitable method has been developed for the extraction, enrichment and separation of the volatile amines present in various tomato products. The method is suitable for other food products as well, only the initial sample size has to be adjusted to the expected amine concentration. 11 volatile amines could be identified in the fresh tomato and tomato powder samples, while 12 amines were found in the tomato purée sample. The estimated volatile amine content of the most concentrated sample, fresh tomatoes, has been found to be in the 1 ppm range. Processing decreases the volatile amine content, it is halved in tomato purée and further decreased, to about a third of the purée level, in tomato powder. Significant differences were observed in the concentrations of the i-butyl- and i-pentylamines. They accounted for about 90% of the volatile amine content in fresh tomatoes, but decreased significantly in the tomato purée and powder samples. Identical amines were identified in all three samples, except for n-pentylamine which could be detected only in the tomato purée sample.

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INVESTIGATIONS INTO THE MICRONUTRIENT DEFICIENCY OF NORTH KARELIAN BEDROCKS WITH REGARD TO HEALTH PROBLEMS OF THE POPULATION

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Microanalytical determinations of the Mn, Cu and Zn content of the bedrocks of North Karelian soils demonstrate a sufficient supply of Zn, less than the usual amount of Cu and a significant deficiency of Mn in comparison with world averages of the continental crusts. The occurrence of cardiovascular diseases is here the largest in the world. The geographical boundaries of these two phenomena coincide. Micronutrient content of the bedrock might influence the content of the soil, plants, animals, the whole food chain of human nutrition. If some causal relationship exists between these facts then Zn supply cannot be a contributing risk factor in cardiovascular diseases, however, Cu deficiency is not excluded and Mn deficiency is the most pronounced in this geochemical territory. This suspicion must be confirmed by more direct proofs, for which these investigations offer some guiding line.

The occurrence of cardiovascular diseases is the highest in the world in Finland. North Karelia (East-Finland) is particularly at a disadvantage within Finland in this sad aspect, little over half of all death occurrences being caused by cardiovascular diseases. This problem has attracted international attention and investigations (Keys, 1970; Bolander, 1971; Puska, 1973).

This population, inhabiting small towns, villages and farms is hard-working, generally lean, and is living a balanced physically active life in a healthy, natural habitat. Furthermore those persons suffering from cardiovascular diseases are not overweight. In spite of these favourable circumstances, a large part of the population has anomalously high blood pressure, cholesterol level and faces a life expectancy of many years shorter than normal (Keys, 1970; Bolander, 1971; Puska, 1973). The suffering of so many caused by disability, permanent fear of a sudden death, shortened life expectancy, motivate the effort made within this study.

As the geology and soils of this territory are different from many other parts of Finland and most of other countries, these geochemical investigations are aimed at the basement rocks of the soil, with the suspicion that – in addition to the generally recognized risk factors – some deficiency might exist. Something essential for health might be missing from or deficient in the soil and food chain and might contribute an additional risk factor for cardiovascular diseases.

The bedrock of the territory consists of presvecokarelidic basement complex, aged over 2500 my, and karelidic metasediments mainly mica schists aged 1800–2200 my. The basement gneiss complex consists mainly of grano- and quartz-dioritic gneisses and a little younger potassium-granites. Also some small metaschists are known inside the basement complex. The whole territory was covered by ice up till the end of the last glaciation (Würmian) and all older sedimentary soils have been entirely eroded by the glaciers. All the withered bedrock has also been eroded by the glaciers. The present soil is composed of glacial till and some glaciofluvial sediments which are in places covered by peat. Perhaps about 30% of arable soils consists of peat. The soil is quite thin, only 3–7 meters on the average. Suspicion might fall on the micronutrients which are well-known to be necessary for health and life in veterinary and human sciences. The deficiency of Cu is known to cause cardiovascular problems in animals (UNDERWOOD, 1971).

Concerning the more important micronutrients (Fe, Mn, Cu, Zn, J, F, Co, Mo *etc.*) the role of several of them is already well known (UNDERWOOD, 1971) and the deficiency in human beings caused by them can be easily diagnosed, recognized. However, many roles of Mn, Cu and Zn are still not sufficiently explored, although it is known that they are coactivators of many metalloenzymes. Particularly little is known about the role of manganese.

1. Materials and methods

A number of samples were collected from the soilforming basement rocks from the whole territory and analysed for Mn, Cu and Zn content. About 25–30 g of each rock sample was crushed and ground to a fine powder in an agate mill. Two-g samples have been taken from this well-mixed powder and dissolved in platinum crucible by hydrofluoric and perchloric acid. The residue was evaporated twice with *cc.* hydrochloric acid, then dissolved in 0.1 *N* HCl and after perfect dissolution the Mn, Cu and Zn contents were determined by an atomic absorption spectrometer, in air-acetylene flame.

Errors of the individual measurements do not exceed $\pm 5\%$.

The following wavelengths were used: Mn: 279.5 nm; Zn: 213.8 nm; Cu: 324.7 nm. The integration periods were in every case: 25 s.

2. Results

Places of sampling are denoted on the geological map (Fig. 1).

The overwhelmingly occurring 3 types of rocks are distinguished by different marks and by serial numbers. The number of collected samples is

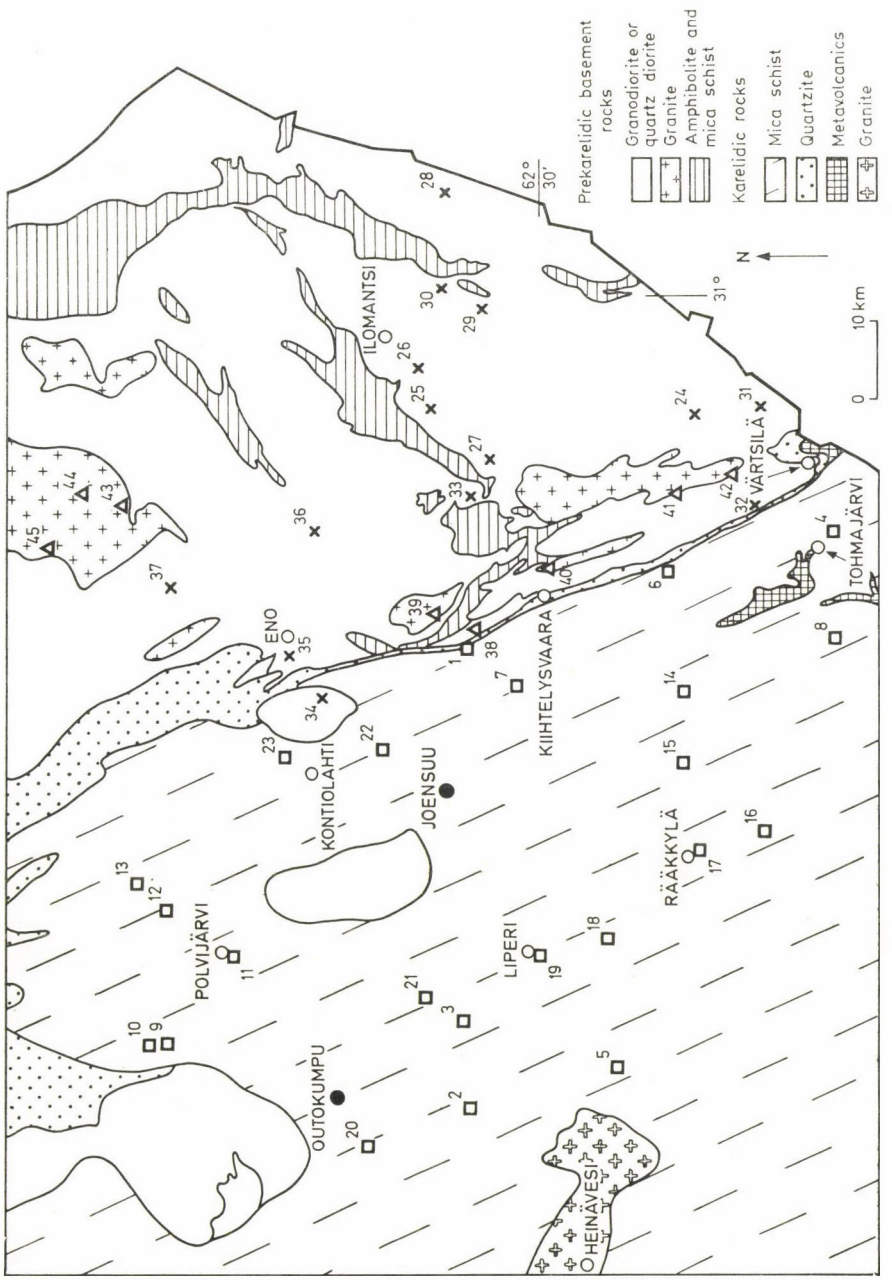

Fig. 1. Simplified map of North Karelia, with sampling places denoted by numbers and different marks for the 3 most abundant types of bed-rocks

Table 1

Micronutrient content of North Karelian rock samples

Sample No.	Sampling place	Mn	Cu	Zn
		(in ppm, *i.e.*: mg per kg)		
Mica schists (Marked on the map by □)				
1	Kiihtelysvaara Hejnävaara	409	12.3	58
2	Liperi Korpivaara	494	37.9	73
3	Liperi Kompero	565	41.8	85
4	Tohmajärvi Uusi-Värtsilä	411	103.7	164
5	Liperi Lammu	446	28.5	79
6	Kiihtelysvaara Holmansuo	671	147.1	107
7	Pyhäselkä Suoranta	580	98.0	138
8	Tohmajärvi Ruppovaara	483	31.9	74
9	Polvijärvi, Lehtovaara	475	16.9	86
10	Polvijärvi, Lehtovaara	360	15.7	73
11	Polvijärvi Kirkonkyla	387	26.5	118
12	Polvijärvi Huutokoski	438	27.8	85
13	Polvijärvi Ruvaslahti	463	36.2	93
14	Tohmajärvi Onkamo	525	14.3	102
15	Rääkkylä Sopensuo	403	40.9	66
16	Rääkkylä Koivusalo	321	30.8	77
17	Rääkkylä Kirkonkylä	390	22.1	59
18	Liperi Salonnenä	215	9.5	66
19	Liperi Kirkonkylä	365	11.6	64
20	Outokumpu Kuusjärvi	412	31.8	62
21	Liperi Viinijärvi	407	36.9	109
22	Kontiolahti Kuurna	150	12.8	44
23	Kontiolahti Latvajärvi	942	17.3	95
Mean values of 23 mica schist samples		448.3	37.1	85.9
Standard error		±32.2	±7.1	±5.8
Granodiorites (Marked on the map by X)				
24	Tuupovaara Ristivaara	720	13.5	104
25	Ilomantsi Koukkuvaara	335	17.3	63
26	Ilomantsi Maukkula	450	7.5	59
27	Tuupovaara Jokilampi	208	8.1	42
28	Ilomantsi Möhkö	737	4.3	106

Table 1 (contd.)

Sample No.	Sampling place	Mn	Cu	Zn
		(in ppm, *i.e.*: mg per kg)		
29	Ilomantsi Haukivaara	620	50.2	93
30	Ilomantsi Meskenvaara	871	33.8	121
31	Värtsilä Ahvenvaara	174	20.0	35
32	Tohmajärvi Saario	159	11.8	34
33	Tuupovaara Kortevaara	675	19.4	124
34	Kontiolahti Jakokoski	560	15.2	82
35	Eno Novikka	269	6.1	42
36	Eno Majoinvaara	290	6.1	42
37	Eno Uimaharju	319	12.4	81
Mean values of 14 granodiorite samples		456.1	16.1	73.4
Standard error		±63.4	±3.3	±8.6
Granites (Marked on the map by △)				
38	Kiihtelysvaara Heinävaara	83	10.8	28
39	Kontiolahti Kansanaho	71	15.2	13
40	Kiihtelysvaara Palo	171	3.5	35
41	Kiihtelysvaara Uskali	130	4.3	33
42	Tohmajarvi Kutsu	146	16.9	38
43	Ilomantsi Kivilahti	298	7.0	47
44	Ilomantsi Oinasvaara	28	4.6	12
45	Lieksa Joakonvaara	172	9.2	34
Mean values of 8 granite samples		137.3	8.9	29.9
Standard error		±29.2	±1.8	±4.3
Mean value of all 45 North Karelian samples		395.5	25.5	72.0
Standard error		±31.2	±4.1	±4.9
World averages of				
Granites		400	10	40
Basalts		1500	100	100
Continental crust resp. sedimentary rocks		950	55	70

approximately proportional to the areal extension of the rock types. Serial numbers, sampling places are represented in Table 1 with values of micronutrient contents in ppm (*i.e.* mg kg^{-1}). Mean values are calculated and denoted for the three types of rocks separately and for the whole territory as well. The standard deviation of the mean values represents the natural variance of rocks, the errors of determination being smaller.

The world averages of the Mn, Cu, Zn content of rocks, known from literature (TUREKIAN & WEDEPOHL, 1961; RADER *et al.*, 1962; VINOGRADOV, 1962; TAYLOR, 1964) are represented under the North Karelian data in Table 1.

If we compare the mean values of the listed data, the following can be said:

The Zn content seems to be about the same as the world average of the continental crusts (resp., sediments). Comparing the content of the granite samples with the world average of granites, it is about the same.

The Cu content of granite samples is about the same as the world average of granites and the mean value of all samples is less than the world average of the continental crust.

The Mn content of granite samples is only about 1/3 of the world average of granites and the mean value of all samples is less than the half of the continental crust.

If we assume that the micronutrient supply may have some significant contribution to the high occurrence of cardiovascular diseases in North Karelia then such a contribution by deficiency is definitely excluded in the case of Zn, it is not excluded in the case of Cu, however, it is more probably in the case of Mn.

Many investigations have been made in the USSR concerning fertile soils. KATALYMOW (1969) mentions an average Mn content of about 1000 ppm in fertile plain land soils of the USSR (KATALYMOW 1969). It is well known from geology that in the course of magmatic separation the silica-rich acidic granite fraction is very impoverished in Mn content and the most alkaline fraction, basalt is enriched in it, the approximately neutral diorites stay in the middle of this separation sequence. Sedimentary soils covering the continents and being the place of agricultural activity, originate from the detritus of some or a mixture of them. Generally speaking soils originating in basalt and andesite are rich in it. Basaltic rock occurrences are very small in North Karelia compared with other rock types. Mn content of the North Karelian granites seems to be even much less then the world average of granites. Contemplating all these facts the following seems to be scientifically well established:

North Karelia is generally a manganese deficient geochemical province.

The food chain leading to the alimentation of mankind has its basis in the bedrock and soil on which the plants are grown. When already the bedrock and soil are deficient then a deficiency of the whole food chain through the

pathway plant – animal – man can occur. It must be emphasized that the investigations of the senior author (SZALAY *et al.*, 1975) carried out earlier in peat fields in Hungary, demonstrated that peat retains manganese and to a smaller extent copper as well, by cation exchange sorption from the subsoil water and the plants grown on peat soils suffer more or less deficiency even where the underlying mineral soil and peat itself contain sufficient amounts of Mn and Cu. This observation was confirmed by several thousand analyses carried out on 13 geographically independent peat fields in Hungary. The cation exchange sorption of Mn and Cu on the peat soil might impair the Mn supply of plants grown on peat soils of North Karelia. However, it must be mentioned here that some Cu deposits of economic grade are known in North Karelia.

3. Conclusions

The high occurrence of cardiovascular diseases among the population of North Karelia and the Mn deficiency of the North Karelian bedrocks and soils seem to be scientifically well established two separate facts, occurring in the same geographically very defined distinct area. (Map on Fig. 1.)

It must be emphasized however, that the causal correlation between them is merely a reasonably based new hypothesis for which more arguments and direct proof are necessary.

In the complex food chain from soil to plants to animals and to man the micronutrient deficiency of bedrocks is but one significant factor. The intermediate links of the chain can contribute further in a negative or positive sense. Only a small part of the total micronutrient content of soils is mobile, available for plants, but no reliable analytical methods exist to determine this part separately. Imported part of food, nutritional habits *etc.* must also be considered. The micronutrient content of the most important food components must be determined and the contribution calculated. It will be reported about these later, as the aim of the present paper was only to clarify the geochemical background of this problem.

It should be only additionally mentioned here that dairy farming proliferates in this territory and dairy products and meats supply abundant amounts of Zn but very little Mn to the population.

*

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BOOK REVIEW

RUDOLF HEISS: **Verpackung von Lebensmitteln. Anwendung der wissenschaftlichen Grundlagen in der Praxis**

Springer Verlag, Berlin, Heidelberg, New York, 1980. 306 pages.

Professor Heiss, for many years the Head of the Institute for the Technology and Packaging of Food of Technical University of Munich, compiled all the experience he has gained on the packaging of food with special attention to the realization of scientific results.

The book is divided into eight chapters and an index. One hundred and five figures illustrate the text. The references, more than 360 altogether, complementing the text, give an overall picture of research results, published mostly in German.

In Chapter 1 the environmental protection aspect of food packaging is discussed. Chapter 2 deals with the climatic requirements for packaging, Chapter 3 describes the packaging requirements raised by commercialization, storage and transport. The properties of packaging materials used for foods, such as plastics, paper, glass, aluminium and other metals are discussed in Chapter 4. In Chapter 5 the protecting capacity of the various packaging materials and packages, in other words their permeability to water, vapour, gas, odour, liquid and light is described. The protection of foodstuffs particularly sensitive to vapour, oxygen or light is analysed in Chapter 6. In Chapter 7 the interaction of packaged foods and packaging materials is discussed from the point of view of food legislation in the German Federal Republic. Chapter 8 presents a general treatment of the utilization of packaging materials, their processing into protecting wrapping.

The book is of assistance to those who are involved in food packaging, however, it may be useful to anybody engaged in packaging. The theoretical considerations and their practical conclusions are of interest to researchers, to those engaged in quality control, to food technologists as well as to university students.

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